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胎兒時期汞暴露與兒童成長及發展

In-utero Exposure to Mercury on Child Growth and Development

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本論文係黃雪倫君（D98841005）在國立臺灣大學職業醫學與工業衛生研究所完成之博士學位論文，於民國 103 年 9 月 24 日承下列考試委員審查通過及口試及格，特此證明

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In-utero Exposure to Mercury on Child Growth and Development

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中文摘要

背景及目的：甲基汞是環境中最常見的一種有機汞，已被證實具有神經毒性和致畸胎毒性。在過去的動物實驗以及甲基汞汙染事件發現發育中的胎兒對於甲基汞毒性相較於成人更為易感。食用較大型的海水魚為人類暴露到甲基汞最主要的暴露途徑之一。不過，在一般吃魚族群的研究當中，胎兒時期甲基汞對於兒童神經發展與發育之影響的發現並不一致。而導致結果不一致的其中一個主要原因，被認為是因為海水魚類富含多種有益於胎兒和兒童成長發育的營養成分，例如：多元不飽脂肪酸、維生素 A、B、D 等，稀釋或抵銷了甲基汞的危害。然而，這並非唯一的解釋。易感基因在不同族群的分佈差異也可能是導致結果不一致的原因。本研究的目的為探討胎兒時期汞暴露與不同 APOE 基因多型性對兒童神經行為發展的影響。另外，探討胎兒時期汞暴露對嬰幼兒生長之長期影響以及討論其在不同性別之影響上有無差異。

方法：本研究之對象為在 2004 年 5 月至 2005 年 1 月間，參與台灣出生長期追蹤研究之 486 對母嬰配對。我們在產前以結構式問卷訪視母親，以了解母親懷孕期間之暴露和基本人口學資料。在生產時收集臍帶血，以感應藕合電漿質譜儀（Inductively Coupled Plasma Mass Spectrometry，ICP-MS）測量臍帶血中之總汞濃度，了解胎兒時期汞暴露的情況。同時，並於病歷上摘錄出生結果。在孩童兩歲時，由專業人員以嬰幼兒綜合發展測驗（Comprehensive Developmental Inventory for Infants and Toddlers，CDIIT）為工具進行兒童發展評估。且請家長填寫兒童行為檢核表 （Child Behavior Checklist，CBCL）作為兒童行為之評估。兒童生長資料則是摘錄自兒童健康手冊上紀錄之資料和兒童餐與０－９歲間追蹤活動時之所實際測量之資料。

結果：在探討不同的 APOE 基因多型性對於汞在兒童發展之影響的研究中，我們發現，在調整前和調整後，只在帶有 APOE ε4 基因的孩童中有發現臍帶血中的汞濃度對兒童的在 CDIIT 所有分項測驗皆有發現一致性不良的影響。在兒童行為的方面，將暴露為高低兩組後，我們發現，帶有 APOE ε4 基因型的孩童有較差（分

數越高越不好）的整體的和分項的行為表現總分。進一步在帶有 APOE ε4 基因型且臍帶血汞濃度大於 12 μg/L 的孩童中，相較於對照組（不帶有 APOE ε4 且臍帶血汞小於 12 μg/L），這些孩童的兒童行為檢核表的總分，內在行為總分、情緒反應總分和焦慮／憂鬱總分高於照組且達統計上的顯著差異。在兒童的生長的部分，我們發現臍帶血汞濃度對和兒童在兩歲的體重、身高和身體質量指數（Body mass index， BMI）存在負相關，且在女生看到的影響較為顯著。

結論：本研究發現只有帶有 APOE ε4 基因型的孩童中，臍帶血汞濃度對於兒童神經行為發展有負相關。臍帶血中的汞濃度對女孩出生體重和身長觀察到顯著的負相關。另外，在生長部分發現和孩童兩歲的體重成負相關，且在女孩較為明顯。胎兒時期的汞暴露對兒童的生長的影響有性別上的差異，對女生生持續到 9 歲仍有發現負相關。此部分需要更多的研究來證實。

關鍵字：汞、甲基汞、APOE 基因多型性、出生結果、兒童生長、神經行為發展

Abstract

Background. Mercury (Hg) is recognized as a neurotoxin and teratogen. However, prenatal methyl mercury (MeHg) exposure on neurodevelopment in the fish-eating population remains controversial. The benefit of the nutrition elements of fish could overcome or offset the adverse effect of MeHg, but this process may not be the only reason for explaining the controversial finding. The varying frequency of susceptible genes amongst these populations may be possible explanations for these observations. Additionally, prenatal mercury exposure was associated with the first two years of postnatal growth. The length of this negative impact of prenatal Hg exposure on child growth and a potential gender difference in mercury exposure susceptibility are unknown.

Objectives. First, we intended to examine and investigate the role of genetic polymorphism of apolipoprotein E (APOE) in various neurodevelopmental and neurobehavioral outcomes. Second, we aimed to investigate the relationship between cord blood Hg concentration and child growth from birth to 9 years of ages and to determine whether a gender difference exists regarding susceptibility to prenatal Hg exposure.

Methods. The study population was 486 mother-infant pairs who gave birth in Taiwan between August 2004 and January 2005 from the Taiwan Birth Panel Study. We interviewed the participants using a structured questionnaire before delivery and

collected umbilical cord blood at birth. Mercury levels in umbilical cord blood were analysed using ICP-MS, and the detection limit of this method was 0.18 ng/mL. Genetic polymorphisms of APOE were analysed using Polymerase Chain Reaction–Restriction Fragment Length Polymorphism. We followed neurodevelopment and behaviour using the Comprehensive Developmental Inventory for Infants and Toddlers (CDIIT) and the Child Behaviour Checklist (CBCL) in subjects two years of age. Growth data from birth to 9 years old were extracted from Child Healthcare Handbooks and follow-up studies. Results. Regarding the modification effect of APOE to mercury on neurodevelopment, the results showed that adverse effects on neurodevelopment were consistently associated with prenatal Hg exposure in all subtests of CDIIT among ε4 carriers as assessed by both simple linear and multiple linear regression models. An interaction between gene polymorphisms of APOE and Hg levels was found. Regarding child behaviour, we found that an increase in cord blood Hg concentrations in APOE ε4 carriers was consistently associated with poorer behaviour performance. The group of ε4 carriers with an elevated cord blood Hg concentration had significantly higher scores in the syndrome categories of general internalizing, emotionally reactive, and anxiety/depression, as well as CBCL total scores. Regarding birth outcomes and postnatal growth, we found that the adverse effect of prenatal Hg exposure was associated with child body weight and body mass in the first 2 years of life in girls.

Prenatal Hg concentration was associated with decreased head circumference from birth to six months of age and body weight, body length and body mass in female children aged 0 to 2 years old.

Conclusion. We suggest that APOE may modify the toxicity of MeHg. APOE ε4 carriers may be more vulnerable to the effects of MeHg on neurodevelopment and child behaviour at two years of age. We suggested that girls were more susceptible to prenatal Hg exposure, which negatively affected their birth and postnatal weight.

Keywords：Mercury, Methyl mercury , APOE, birth outcome, child growth, neurobehavioral development

Abbreviation: Mercury, Hg; Methyl mercury, MeHg; Apolipoprotein E, APOE; Comprehensive Developmental Inventory for Infants and Toddlers, CDIIT; Child Behavior Checklist, CBCL

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Chapter One: General Introduction

1.1 Background

Mercury (Hg), a heavy metal, is a confirmed teratogen and neurotoxin that is widespread throughout the environment. Hg exists in the environment in natural and anthropological processes. There are three major forms of mercury: organic, metallic and elemental. Methyl mercury (MeHg) is the primary and most toxic form of mercury to which humans are exposed. MeHg contains both characteristics of high bioaccumulation and bio-magnification; therefore, it is widespread and is one of the most threatening and persistent pollutants in our environment. MeHg is approximately 95 to 100% absorbed by the gastrointestinal tract (Clarkson, 2002). MeHg is able to pass through the placental blood barrier and foetal blood brain barrier, then accumulate in the brain tissue, and adversely affect foetal brain development or cause brain damage (Cernichiari et al. , 1995, Clarkson and Magos, 2006, Farina et al. , 2011, Yee and Choi, 1996). An in vivo model indicated that MeHg induces oxidative damage in neural cells by generating free radicals that cause mitochondrial damage, lipid peroxidation, and microtubule destruction (Yee and Choi, 1996). An animal study that mimicked the exposure status of humans demonstrated the deleterious effects of mercury on motor abilities, coordination, overall activity, and mnemonic functions (Montgomery et al. , 2008). Hg binding to haemoglobin with high affinity is a factor that explains the higher mercury concentration in cord blood compared to maternal blood (Sakamoto et al. ,

2004). As the developing brain is more susceptible to the toxic effects of MeHg, prenatal MeHg exposure is a serious public health issue.

1.2 International comparison of cord blood mercury concentration

The median concentrations of Hg in maternal and cord blood were 6.3 μg/L and 12.3 μg/L, respectively, as reported in the Taiwan Birth Panel Study, which were lower concentrations than those reported in New Zealand (Crump et al. , 1998), the Faroe Islands (Dahl et al. , 1996, Grandjean et al. , 1998, Grandjean et al. , 1997)and the Seychelles (Marsh et al. , 1995) and was higher than those reported in China (Gao et al. , 2007), Korea (Lee et al., 2010), the United States (Lederman et al. , 2008, Oken et al. , 2005), and Poland (Stewart et al. , 2002). Fish consumption is the primary exposure pathway of Hg in Taiwanese women of child-bearing age (Chien et al. , 2010, Hsu et al. , 2007). In the TBPS study, 90% of the study subjects ate marine fish, and 96% ate seafood during pregnancy. The cord blood Hg levels in our study were comparable to those reported in previous studies in Taiwan.

1.3 Mechanisms

Several mechanisms associated with MeHg induction of developmental and neurotoxicity have been observed, including inducing oxidative stress (Ou et al. , 1999), interfering with homeostasis of calcium (Sirois and Atchison, 2000) and glutamate (Aschner et al. , 2000), altering depletion of glutathione (Shanker et al. , 2005) and

inhibiting the thioredoxin system. It is well recognized that sulfhydryl groups (thiols) play an important role in Hg toxicity. Both glutathione and thioredoixin systems play important roles in mercury toxicity. Glutathione is the most abundant intracellular thiol containing molecule in human cells. MeHg can alter the activities of the glutathione-related enzymes, glutathione peroxidase and glutathione reductase (Farina et al. , 2005, Farina et al. , 2003, Stringari et al. , 2006). The levels of glutathione may decrease through the interaction with Hg (Shanker, 2005). The depletion of glutathione may increase the levels of reactive oxygen species' oxidative stress and contribute to mitochondrial dysfunction (Ou, 1999). Furthermore, a previous study has demonstrated that the prenatal exposure to MeHg may affect postnatal development of antioxidant systems. This study provided the possible molecular mechanism underlying the harmful effect of prenatal MeHg exposure during the critical period of development, which may trigger long-lasting functional deficits in the developing brain (Stringari et al. , 2008). Thioredoxin reductase, like glutathione peroxidase, is responsible for catalysing redox reactions involved in fundamental mechanisms of cell growth, such as DNA synthesis and antioxidant defence, and is critical for cellular stress response, protein repair, and protection against oxidative damage (Carvalho et al. , 2008, Papp et al. , 2007, Rozell et al. , 1985). A recent *in vivo* study suggested that Hg can inhibit the mammalian thioredoxin system and could be a major molecular mechanism of mercury toxicity

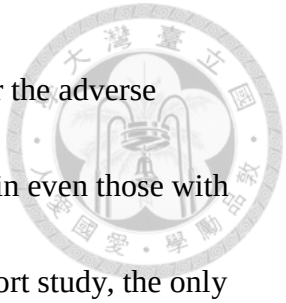
(Carvalho, 2008). Because thioredoxin reductase and thioedixin are widely distributed in various mammalian organs and tissues, the potential global toxicity of Hg should be considered, in addition to neurotoxicity.

1.4 Prenatal mercury exposure and birth outcomes

The impacts of prenatal mercury exposure on birth outcomes in fish eating populations remain unclear. Previous studies that have investigated the relationship between prenatal Hg exposure and birth outcomes have shown mixed results. A recent Spain cohort study demonstrated that cord blood mercury was negatively associated with cord blood Hg levels after data were adjusted for covariates, including fish consumption (Ramon et al., 2009). However, no association between birth weight and prenatal Hg exposure was demonstrated in cohort studies in France (Drouillet-Pinard et al. , 2010), New York (Lederman, 2008), Canada(Lucas et al. , 2004) and the United Kingdom (Daniels et al. , 2007). Currently, there is no sufficient evidence regarding the effect of Hg on anthropometric measures at birth.

1.5 Prenatal mercury exposure and children neurodevelopment

Methyl mercury (MeHg) is recognised as a neurotoxin, and the developing brain is more susceptible and vulnerable to the toxic effects of environmental toxicants than the adult brain (Clarkson and Magos, 2006, Farina, 2011, Franco et al. , 2009, Yee and Choi, 1996). However, the adverse effect of prenatal Hg exposure on neurodevelopment



in the fish-eating population remains controversial, and the reason for the adverse effects remain unclear. These inconsistent findings were represented in even those with a relatively high level of prenatal Hg exposure. In the Seychelles cohort study, the only confirmed deleterious effect on "motor speed and coordination" in the non-dominant hand was observed in 9-year-old Seychellois children (Davidson et al. , 2006b, Myers et al. , 2003), but this adverse effect was not shown among 10- and 11-year-olds (Davidson et al. , 2008, Davidson et al. , 2006a). A later cohort study conducted in the Seychelles demonstrated a consistent adverse effect on neurodevelopment at ages 9 and 30 months if the beneficial effects of fish were included in the statistical analysis (Debes et al. , 2006, Stokes-Riner et al. , 2011, Strain et al. , 2008), suggesting that the beneficial nutritional effects may have obscured the adverse effect of Hg. In contrast, in a cohort study from the Faroe Islands, where the prenatal Hg level is lower than the level reported in the Seychelles, consistent adverse effects on the motor, attention and language function were found at ages 7 and 14 years without adjusting for fish consumption(Debes, 2006, Grandjean, 1997).

1.6 Prenatal mercury exposure and child behavior

Animal studies designed to mimic human gestational MeHg exposure found a significant decrease in motor and mnemonic capabilities in exposed mice as well as a minor increase in attention deficit and anxiety concentrations (Liang et al. , 2009, Montgomery, 2008). An adverse impact of prenatal exposure to MeHg through fish consumption on children's behaviour (Boucher et al. , 2012, Sagiv et al. , 2012, Debes, 2006) has been reported in studies at different degrees of prenatal MeHg exposure in various countries. Two cohort studies in North America found that Hg concentrations were associated with increased inattention and aggressive behaviour in school children (Boucher, 2012, Sagiv, 2012). However, no association was found in the Seychelles cohort study, even though the Hg exposure concentrations in the Seychelles were higher. In Seychelles cohort studies, in which study subjects were prenatally exposed to a moderate level of MeHg (approximately 6 μg/g in maternal hair), no association between prenatal MeHg exposure and children's cognition and problematic behaviour was reported (Davidson et al. , 1998, Davidson, 2006b, Myers, 2003, Myers et al. , 2000). A primary explanation of these inconsistencies is the compensatory effect of nutrients in seafood that overcome the neurotoxicity of MeHg(Sakamoto, 2004, Stokes-Riner, 2011, Strain, 2008).

1.7 Prenatal mercury exposure and postnatal growth

A Denmark cohort study first demonstrated that cord blood mercury concentration was associated with body weight of 18 month old infants (Grandjean et al. , 2003). A recent South Korean cohort study also reported that late pregnancy and cord blood Hg were associated with infant weight gain at 12 months and 24 months of age without considering fish consumption (Kim et al. , 2011). However, the duration of this negative impact of prenatal Hg exposure on child growth remains unclear.

1.8 The micronutrients in fish may overcome the adverse effect of mercury on child neurodevelopment

The inconsistent finding on mercury exposure and child neurodevelopment may partially be due to the beneficial nutrients found in fish, such as unsaturated fatty acids (Sakamoto, 2004, Stokes-Riner, 2011, Strain, 2008), selenium and other micronutrients. A negative effect on visual recognition memory at 6 months of age (Oken, 2005) and poorer performance on the Peabody Picture Vocabulary Test (PPVT) and the Wide-Range Assessment of Visual Motor Abilities (WRAVMA) at age 3 years were reportedly related to cord blood Hg levels in Massachusetts residents(Oken et al. , 2008). Although the cord blood Hg level reported in New York City residents was higher and the adverse effect of Hg on neurodevelopment was obvious and consistent in the Massachusetts study, no significant adverse effect on neurodevelopment was found in

the New York City children at ages 12 to 24 months or 36 months to 48 months (Lederman, 2008). Therefore, the benefit of fish was also insufficient to explain these inconsistent findings within a country. Additionally, Jedrychowski and colleagues reported that adverse effects on both the Psychomotor Development Index (PDI) and the Mental Development Index (MDI) were found in children aged 12 months in Polish children without adjusting for fish consumption(Jedrychowski et al. , 2006) at a relatively lower exposure level than in the US study. Furthermore, those authors reported no association at ages 24 and 36 months, even with adjusting for fish consumption (Jedrychowski et al. , 2007a). Jedrychowski and colleagues then demonstrated that the cord blood Hg level may not accurately represent the maternal exposure level without appropriate correction (Jedrychowski et al. , 2007b). These conflicting findings may be explained by the MeHg and the level of nutrients may vary by the fish species. Many of the studies, however, considered the frequency of fish consumption instead of the level of the nutrients in the fish. This factor may explain these inconsistent findings.

1.9 The role of Apolipoprotien E (APOE) on Hg related neurotoxicity

Marine fish is one of the primary sources of mercury exposure, and its nutritional benefit may partially explain the conflicting findings in adverse effects that have been widely discussed. However, few studies have investigated the role of genetic

susceptibility in the relationship between Hg exposure and child neurodevelopment. APOE is as a crucial factor involved in cholesterol metabolism, neuron growth and neuron repair in the central nervous system (Buttini et al. , 1999, Mahley, 1988). Cholesterol and fatty acids are essential for brain development. Therefore, it is rational to assume that the genetic polymorphisms regulating fatty acids and cholesterol may modify the effect of Hg on child neurodevelopment.

APOE has three common variants: epsilon2 (ε2), epsilon3 (ε3) and epsilon4 (ε4) (Zannis et al. , 1982). Different APOE alleles may vary the susceptibility of an individual to MeHg-related neurological disorders. Several studies have reported that the ε4 variant was associated with a neurodevelopmental advantage in children who had unfavourable exposure or nutrient deficiency (Oria et al. , 2005, Wright et al. , 2003). A possible explanation is that ε4 alleles are associated with elevated cholesterol absorption in the intestine and may be potentially advantageous in early neurodevelopment (Oria, 2005, Weisgraber et al. , 1982). However, the ε2 and ε3 alleles contain at least one cysteine and an amino acid that contains a thiol functional group, which mediates the elimination of MeHg at positions 112 and 158, whereas the ε4 allele does not have a cysteine at either site that might be associated with increased Hg levels. Additionally, the ε4 allele was also found to have limited neuron repair ability (Buttini, 1999, Herz and Beffert, 2000, Mahley, 1988, Ohkubo et al. , 2001), and it may be associated with a

higher risk of developing neurological diseases and an increased susceptibility to Alzheimer's disease (Godfrey et al. , 2003). A previous longitudinal study suggested that an APOE ε4 carrier might be associated with hyperactivity, emotional reactions and sociability problems in children and young adults (Keltikangas-Jarvinen et al. , 1993). Whether APOE ε4 variant carriers are more susceptible to neurological disorders or confers an advantage against mercury exposures on children neuronal health remains unclear.

1.10 Research Designs

This study was an investigation of the Taiwan Birth Panel Study (TBPS), and a total of 486 pregnant women and their neonates were recruited between April 2004 and January 2005 from one medical hospital in Taipei city, one area hospital, and two clinics in Taipei County. Before enrolling the study participants, informed consent was obtained before delivery to collect maternal and umbilical cord blood and prenatal questionnaires. For all the 486 pairs, we collected maternal blood (10 ml) and cord blood (10 ml) at delivery. The bloods were separated into whole blood, plasma and DNA. The plasma samples were stored at -80℃ until being processed for laboratory analysis. A structured questionnaire was interviewed within three days after delivery to characteristics, and history of active and passive smoking during pregnancy was collected(Hsieh et al. , 2011).

We continued to follow our study participants at different ages to collect postnatal health outcomes. We followed their health related information, including medical history, life style related behaviour, environmental exposure and parenting related information by mail questionnaire at 4, 6, 12, 24, 36, 60, 84 and 108 months of age. Within three days of age, the neonate neurobehavioral examination (NNE) was performed to assess early children neurodevelopment. At 6 and 24 months of age, a Comprehensive Developmental Inventory for Infants and Toddlers (CDIIT) test and Infant/Toddler HOME of Home Observation for Measurement of the Environment Inventory (IT-HOME) were performed to assess early childhood neurobehavioral performance. The Child Behaviour Checklist (CBCL) was followed repeatedly at 24, 36, 60 months of age. The MacArthur Communicative Development Inventory (CDI) was performed to assess early childhood behavioural problems and language development. The intelligence quotients of study subjects were examined using Wechsler Preschool and Primary Scale of Intelligence-Revised (WPPSI-R) at 7 years of age. All protocols used in these follow up periods were approved by the Ethical Committee of National Taiwan University Hospital.

1.11 Cord blood mercury analysis

Cord blood samples were collected in ethylene-diamine-tetraacetic acid (EDTA) tubes, separated into whole blood and serum, and then stored at -80 °C until laboratory

analysis. Blood Hg concentrations were analysed using an Agilent 7500C inductively coupled plasma mass spectrometer (ICP-MS) (Barany et al. , 1997). One spike per 10 samples was applied for quality assurance and quality control. The detection limit of Hg in the present study was 0.18 μg/L, and the cord blood Hg levels of all participants were detectable.

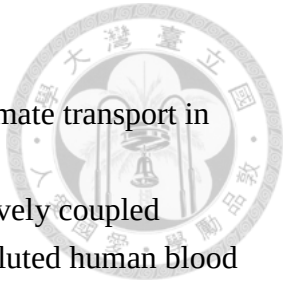
1.12 APOE genetic polymorphism analysis

The Chemagic deoxyribonucleic acid (DNA) specialised blood kit (Chemagen, Aachen, Germany) was used to extract DNA by following the manufacturer's protocol. A 218 base-pair DNA fragment was amplified using the primer pairs F5'-TCCAAGGAGCTGCAGGCGGCGCA and R5'-GCCCCGGCCTGGTACACTGCCA with polymerase chain reaction (PCR) in an ABI GeneAmp™ 2700 system. The following procedure was used: the samples were initialized at 95 ℃ for 10 minutes to denature the DNA. This step was followed by 35 thermal cycles of denaturing (94 °C for 30 seconds) →annealing (65 °C for 45 seconds)→ extension (72 °C for 45 seconds), and ended with 10 minutes at 72 °C for the final extension. The amplified DNA (10 μL) was digested with two units of AflIII and six units of HaeII for 24 hours at 37 ℃ and then analysed in 4% agarose gel with ethidium bromide staining (Zivelin et al., 1997).

1.13 Aims

Based on the prospective birth cohort study, we aimed to explore the impact of prenatal mercury exposure on birth outcomes, children's neurodevelopment and behavioural outcomes, and postnatal growth. For the neurodevelopment and behavioural outcomes, we measured the potential role of APOE on prenatal exposure. The specific aims were:

1. To investigate the role of APOE on child neurodevelopment
2. To investigate the role of APOE on child neurodevelopment
3. To investigate the impact of prenatal mercury on birth outcomes and postnatal growth



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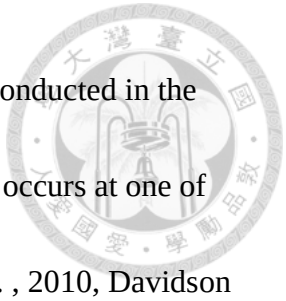
Chapter Two: Mercury, APOE, and children's neurodevelopment

2.1 Introduction

Mercury (Hg) is a confirmed neurotoxin that may have adverse effects on neurodevelopment (Clarkson and Magos, 2006, Farina et al. , 2011, Yee and Choi, 1996). As the developing brain is more susceptible to the toxic effects of Hg, prenatal Hg exposure is a crucial public health issue. Methyl mercury (MeHg) is the primary form of mercury to which humans are exposed. In an animal study designed to mimic exposure to MeHg in fish-consuming populations, murine offspring were exposed to a daily dose of 0.01 mg/kg body weight of MeHg in utero from days 8 to 18 during gestation. The animals experienced deleterious effects on their motor abilities, coordination, overall activity, and mnemonic functions (Montgomery et al. , 2008). However, the impact of prenatal MeHg exposure on neurodevelopment among fish-consuming populations remains controversial. Faroe Island cohort studies have indicated that elevated cord blood Hg levels were associated with adverse effects on motor, attention and language function in children at 7 and 14 years of age (Debes et al. , 2006, Grandjean et al. , 1997). Furthermore, an association between delayed neurodevelopment and cord blood Hg levels has been found in one-year-old Polish children, with median cord blood Hg levels less than 1 μg/L (Jedrychowski et al. , 2006).



In contrast, no adverse effects were reported by three cohort studies conducted in the Seychelles Islands, where prenatal where prenatal exposure to MeHg occurs at one of the highest levels in the world (Davidson et al. , 2001, Davidson et al. , 2010, Davidson et al. , 1998, Davidson et al. , 1995, Davidson et al. , 2006a, Davidson et al. , 2006b, Myers et al. , 2003, Myers et al. , 1995). These inconsistent associations may partially be due to the beneficial nutrients found in fish, such as unsaturated fatty acids (Sakamoto et al. , 2004, Stokes-Riner et al. , 2011, Strain et al. , 2008). Nevertheless, nutritional benefit is insufficient to explain the conflicting findings that adverse effects are found in populations with lower exposure levels, while no adverse effects are found in high-exposure populations, unless the results are adjusted to account for fish consumption. In addition to the beneficial nutrients found in fish, another possible explanation for the conflicting findings may be variations in genetic susceptibility among fish consuming populations. These genetic differences may account for a large proportion of the response to environmental toxins. However, analyses of the gene-environmental interaction regarding the relationship between prenatal MeHg exposure and neurodevelopment are limited. Therefore, the concept of genetic susceptibility was included in this study.

Apolipoprotein E (APOE, protein; Apoe, gene) plays an important role in lipid-transported proteins and it is known to be a crucial mediating factor in neuronal

repair (Buttini et al. , 1999, Mahley, 1988). However, no studies have investigated the role of Apoe in the neurodevelopment of children exposed to Hg. Three common Apoe alleles have been identified: epsilon 2 (ε2), epsilon 3 (ε3) and epsilon 4 (ε4) (Zannis et al. , 1982). Epsilon 2 has two cysteines, ε3 has a cysteine and an arginine and ε4 has two arginines at positions 112 and 158, respectively (Rall et al. , 1982). Cysteine contains a thiol (–SH) functional group, which may conjugate with MeHg and help to remove it from the brain. With the increased risk of Hg accumulation in brain tissue, ε4 carriers may be at a greater risk of developing Hg-induced neurological diseases (Godfrey et al. , 2003). Lead, like Hg, is a known neurotoxin that impairs neurodevelopment in the central nervous system. An occupational study has suggested that neurobehavioral test scores were lower for individuals carrying ε4 allele and that individuals may differ in their susceptibility to the long-term effects of lead exposure (Stewart et al. , 2002). In contrast, a few studies have reported that ε4 carriers display a neurodevelopmental advantage (Oria et al. , 2005, Wright et al. , 2003). To the best of our knowledge, no study has reported Apoe genetic susceptibility regarding prenatal exposure to Hg on neurodevelopment. Therefore, the aim of this study was to investigate the modification effect of Apoe gene polymorphisms on cord blood Hg levels and neurodevelopment in two-year-old children.

2.2 Materials and methods

Study population

A prospective cohort study was conducted between April 2004 and January 2005. A total of 486 mother-infant pairs were recruited from a medical center, a local hospital and two clinics located in northern Taiwan. All of the participants had been informed of the purpose of the research and signed a consent form prior to enrollment. Infant cord blood was collected at delivery, and a structured questionnaire including information regarding family demographics, home characteristics, dietary habits, lifestyle factors and family medical history was collected within three days after delivery (Hsieh et al. , 2008).

Two hundred and eighty-eight participants underwent the Comprehensive Developmental Inventory for Infants and Toddlers (CDIIT) neurodevelopmental assessment during follow up when the infants were two years old. Informed consent was obtained again, and then neurodevelopmental assessment and a composite questionnaire were performed. The study subjects were restricted to those cord blood mercury (Hg), Apoe genotype and neurodevelopment test were assessed. Overall, 181 subjects were eligible for this study. Furthermore, of these 181 subjects, the following subjects were excluded: 2 subjects with ε2/ε4 Apoe genotype, 1 subject had a genetic disease, 10 subjects whose mothers had tobacco smoke during pregnancy (self-reported

or blood cotinine levels >15 μg/L (Peacock et al. , 1998)). Finally, 168 subjects were included in this study. All protocols used were approved by the Ethical Committee of the National Taiwan University Hospital.

Analysis of cord blood Hg

Cord blood samples were collected in ethylene-diamine-tetraacetic acid (EDTA) tubes, separated into whole blood and serum, and then stored at -80 °C until the laboratory analysis. Blood Hg concentrations were analyzed using an Agilent 7500C inductively coupled plasma mass spectrometer (ICP-MS) (Barany et al. , 1997). One spike per 10 samples was applied for quality assurance and quality control. The detection limit of Hg in the present study was 0.18 μg/L and the cord blood Hg levels of all participants were detectable.

The Chemagic deoxyribonucleic acid (DNA) specialized blood kit (Chemagen, Aachen, Germany) was used to extract DNA by following the manufacturer's protocol. A 218 base-pair DNA fragment was amplified using the primer pairs F5'-TCCAAGGAGCTGCAGGCGGCGCA and R5'-GCCCCGGCCTGGTACACTGCCA with polymerase chain reaction (PCR) in an ABI GeneAmp™ 2700 system. The following procedure was used: the samples were initialized at 95 ℃ for 10 minutes to denature the DNA. This was followed by 35 thermal cycles of denaturing (94 °C for 30 seconds) →annealing (65 °C for 45

seconds)→ extension (72 °C for 45 seconds), and ended with 10 minutes at 72 °C for the last extension. The amplified DNA (10 μL) was digested with two units of AflIII and six units of HaeII for 24 hours at 37 ℃, and then analyzed in 4% agarose gel with ethidium bromide staining (Zivelin et al. , 1997).

Measurement of neurodevelopment

The Comprehensive Developmental Inventory for Infants and Toddlers (CDIIT) was used to measure neurodevelopment. The participants were evaluated at two years of age by a trained physical therapist. The developmental data were collected from direct testing, observation, and caregiver-report. The scores were then transformed into developmental quotients (DQs) based on the Taiwanese children's norm.

The CDIIT was established and standardized in Taiwan for assessing the characteristics of children aged 3 to 71 months. The measure consists of cognition, language, motor, social, and self-help subtests, and the motor subtest is divided into gross- and fine-motor subdomains. The validity of CDIIT for the diagnostic of developmental delays was 0.96 in the area under the receiver operating characteristic curve (AUC) (Wang, 2005, Wang and Liao, 2007, Wang et al. , 1998). Furthermore, the test–retest reliability of the intraclass correlation coefficient was 0.76–1.00 (Liao and Pan, 2005), the Cronbach α for internal consistency was 0.75–0.99 and the CDIITs validity compared with the Bayley Scales of Infant Development-II (r = 0.80–0.97) and Peabody Developmental

Motor Scales-Second Edition has been published and is acceptable (Liao et al. , 2005, Wu, 2005).

Other covariates

Lead and environmental tobacco smoke were recognized as environmental risk factors of childhood developmental problems that may potentially interfere in the present study. Therefore, cord blood lead and cotinine levels, which correspond to prenatal exposure to lead and environmental tobacco smoke, were considered in the study. Because the prevalence rate of active smoking during pregnancy was approximately 3 percent in this study, we restricted the potential confounding effect of active smoking during pregnancy by excluding the subjects of mothers with blood cotinine levels greater than 15 μg/L (Peacock, 1998). Additionally, the parental and support environment of the child has been recognized as an important factor that may affect neurodevelopment. Therefore, the Home Observation for Measurement of the Environment - Infant/Toddler version (HOME), which is an assessment tool for measuring the quality and quantity of stimulation and caregiving support provided to a child at home (Caldwell, 2003), was applied to evaluate the home environment of the subjects at two years of age. This assessment was conducted by well-trained interviewers at the subjects' residence. In addition to lead, tobacco smoking data, and the HOME results, other covariate data were reported as interference factors of neurodevelopment, including infant sex, birth

weight, and maternal age at delivery, maternal education level, nationality, annual family income and duration of breastfeeding, were also considered and included in the statistical analysis.

Statistical analysis

The chi-squared test was used to calculate the observed and expected frequencies according to the principal of Hardy-Weinberg equilibrium. The cord blood Hg levels were stratified into two groups based on a cutoff of 12 μg of Hg/L of cord blood, then classified according to 3 *Apoe* genotypes: 1) ε2 carriers: the subjects were ε2/ε2 or ε2/ε3; 2) wild-type or referent-type: the subjects were ε3/ε3; and 3) ε4 carriers: the subjects were ε3/ε4 or ε4/ε4. Table 2-1 presents the subjects' demographic characteristics, and table2-2 shows the subjects' birth characteristic and CDIIT DQs. An ANOVA test was used to determine the differences between the two groups. Blood Hg concentrations were natural-log transformed to conform to a normal distribution.

In table 2-3, we applied both simple and multiple linear regression models to estimate the effects of Hg with and without *Apoe* genotype stratification. In the multiple linear regression, the potential confounders included maternal age, education (junior high school, high school, college and above) and nationality (Taiwan or other), family income when the child was two years old (less than, more than or equal to 1,000,000 New Taiwan dollars), the infant's sex and birth weight (in grams), the HOME score,

and the duration of breastfeeding (less than or equal to two months, more than two months). Lead and cotinine concentrations in the cord blood were natural-log transformed. Considering these factors, the interaction between Hg and *Apoe* gene polymorphism was evaluated. As shown in figure 2-1, the subjects were stratified into three categories according to their genotype, and stratified multiple linear regression models were applied to estimate the beta coefficient of cord blood Hg concentration and each CDIIT subtest with adjustment of confounders.

Two tailed p-values <0.05 were considered to be statistically significant for exploring the relationship between high exposure and low exposure and CDIIT DQs. All analyses were conducted using SPSS 16.0 and SAS Version 9.2 (SAS Institute Inc., Cary, NC).

2.3 Results

One hundred sixty-eight out of 486 subjects conformed to our abovementioned inclusion criteria. At age two, the participants had higher Hg levels than the nonparticipants; however, the participants had better birth outcomes (in terms of birth weight, head circumference and birth length) and a better social economic status: the maternal education level and family income of the followed subjects was higher than that of the nonparticipants (data not shown). The distribution of the *Apoe* genotypes among these groups was comparable. The frequency of the *Apoe* genotypes in the 168 subjects in this study was as follows: ε2/ε2 (0.6%), ε2/ε3 (18.5%), ε3/ε3 (65.5%), and ε3/ε4 (15.5%), which corresponded to the Hardy-Weinberg equilibrium (data not shown).

In table 2-1, the participants were stratified into six groups according to the level of mercury and the *Apoe* genotype. For all groups, the child and maternal characteristics, environmental exposure, socioeconomic status and parenting environment were similar, with the exception of fish consumption during pregnancy. Maternal fish intake during pregnancy was associated with cord blood Hg levels, as shown in table 2-1. In table 2-2, the birth outcomes and neurodevelopment were compared among the six groups, and statistically significant differences were found in the DQ of the CDIIT whole test and the cognition, language, fine motor and social subtests.

Table 2-3 shows that while the *Apoe* gene variations were combined to estimate the effect of Hg level on neurodevelopment, no effect was found in simple linear regression; after adjustment for the confounding factor in a multiple linear regression, a significant positive effect was found for the motor subtest, β=3.95 and 95% confidence interval (CI)=0.51 to 7.39. In the stratified simple linear regression model, Hg level was negatively associated with the DQs of the CDIIT whole test (β=−12.4, 95% CI= −20.79 to −4.02) and the subtests of social (β=−11.68, 95% CI=−22.49 to −0.87), self-help (β=−14.44, 95% CI=−25.37 to −3.51) and fine motor (β=−6.88, 95% CI= −12.97 to -0.79) among ε4 carriers before adjusting for confounders, but no effect was found in non ε4 carriers. After adjustment for confounding factors, a significantly negative association was found between Hg levels and the DQs of cognition (β=−9.31, 95% CI=−14.75 to −3.88) among ε2 carriers. Among ε4 carriers, all of the subtests were consistently negatively associated with Hg levels, and statistical significance was reached for DQs of cognition (β=−8.47, 95% CI=−16.10 to −0.84), social (β=−11.02, 95% CI=−20.85 to −1.19) and the CDIIT Whole test (β=−10.45, 95% CI=−17.36 to −3.54). Furthermore, the interactions between the cord blood Hg level and *Apoe* genotype were tested; for the overall neurodevelopment test, the cognition and motor subtests and the fine-motor subdomain, the p-values of the interaction term were statistically significant ($p<0.05$). After adjustment for potential confounders, the

interaction effect remained for the CDIIT whole test, cognition and language subtests and fine motor subdomain ($p<0.05$). Figure 2-1 shows the consistent negative association between Hg level and neurodevelopment that was found in the subjects who were ε4 carriers.

2.4 Discussion

Mercury (Hg) levels in cord blood were adversely associated with neurodevelopment among ε4 carriers at two years of age, as measured using the Comprehensive Developmental Inventory for Infants and Toddlers (CDIIT). A modifying effect of *Apoe* was considered unless no association or a positive association was found. The interaction between *Apoe* and Hg levels was significant for the CDIIT whole test developmental quotients (DQs) and the DQs of the cognition and language subtests and the fine motor subdomain. Therefore, stratified linear regression was applied to eliminate the modifying effect of the gene. A deleterious effect on cognition was significantly associated with both ε2 and ε4 carriers, in which the DQs of cognition decrements of 9.31 and 8.47 were found per unit (natural log transformation) of increase in Hg levels among ε2 and ε4 carriers. Furthermore, among ε4 carriers, the adverse effect on neurodevelopment was consistently associated with prenatal Hg exposure for all subtests of the CDIIT, and this effect reached statistical significance for the social subtest (β=−11.02, 95% CI=−20.85 to −1.19) and whole test DQs (β=−10.45, 95% CI=−17.36 to −3.54) after adjusting for potential confounders. Overall, the ε4 carriers were more susceptible and vulnerable to prenatal Hg exposure and an interaction between the *Apoe* gene and Hg levels might obscure the deleterious effects of Hg.

The median concentrations of maternal and cord blood Hg were 7.4 μg/L and 12.2 μg/L, respectively, in the present study, which are lower than the concentrations reported for New Zealand (Crump et al. , 1998), Faroe Island (Dahl et al. , 1996, Grandjean, 1997) and Seychelles (Marsh et al. , 1995). However, these concentrations are higher than those reported for China (Gao et al. , 2007), Korea (Lee et al. , 2010), the United States (Lederman et al. , 2008, Oken et al. , 2005), and Poland (Jedrychowski, 2006). Fish consumption is the primary exposure pathway of Hg in Taiwanese women of childbearing age (Chien et al. , 2010, Hsu et al. , 2007). In our study, 94% of the study subjects ate marine fish and 96% ate seafood during pregnancy. The cord blood Hg levels in our study were comparable to those reported in the previous two studies in Taiwan. Additionally, the Hg levels in cord blood are greater than, but strongly correlated ($p<0.0001$) with, the levels in maternal blood, which is consistent with previous reports.

The developing brain is more susceptible and vulnerable to the toxic effects of mercury than the adult brain (Clarkson and Magos, 2006, Farina, 2011, Yee and Choi, 1996). In the present study, Hg levels in the cord blood were approximately two times higher than the benchmark level of 5.8 μg/L as recommended by the US EPA, while adverse effects associated with mercury levels in cord blood on neurodevelopment were only found in ε4 carriers. In the fish-consuming human population, however, inconsistent findings

were recorded even with those with relative high level of prenatal Hg exposure. For instance, in the Seychelles cohort study, the only confirmed deleterious effect on “motor speed and coordination” in the non-dominant hand was observed in 9-year-old Seychellois children (Davidson, 2006b, Myers, 2003), and this adverse effect was not observed in follow-up studies (Davidson et al. , 2008, Davidson, 2006a). A later cohort study conducted in Seychelles demonstrated a consistent adverse effect on neurodevelopment at ages 9 and 30 months when the beneficial effects of fish were included in the statistical analysis (Stokes-Riner, 2011, Strain, 2008), suggesting that the beneficial nutritional effects may have obscured the adverse effects of Hg. In contrast, without adjusting for fish consumption, consistent adverse effects on neurodevelopment were found in 7- and 14-year-old children in a Faroe Islands cohort study (Debes, 2006, Grandjean, 1997). While the nutritional benefits of fish may be one of the reasons for the controversial results reported in the Seychelles study, these benefits are insufficient for explaining the inconsistent findings among the studies.

In addition to populations with high Hg levels in cord blood, the controversial findings of prenatal mercury regarding neurodevelopment in children were also displayed for fish-consuming populations that had lower mercury levels in cord blood, for which the interference effect of fish was controlled. Two studies conducted in Massachusetts and New York City included fish consumptions as a covariate. An obvious and consistent

adverse effect of Hg on neurodevelopment was found in the Massachusetts study, while the cord blood Hg levels reported for the study in Massachusetts were lower than the New York City study (Lederman, 2008, Oken et al. , 2008, Oken, 2005). In contrast, a Polish cohort study reported relatively lower exposure levels than the US study. Adverse effects on both the Psychomotor Development Index (PDI) and the Mental Development Index (MDI) were found in children aged 12 months without adjusting for fish consumption (Jedrychowski, 2006), while no association was found at ages 24 and 36 months, even when including an adjustment for fish consumption (Jedrychowski et al. , 2007a). Furthermore, Jedrychowski and colleagues have demonstrated that cord blood Hg levels may not accurately represent the maternal exposure levels without an appropriate correction (Jedrychowski et al. , 2007b). We hypothesized that gene polymorphism may represent one of the plausible reasons for this difference. We suggested that the genetic susceptibility of the population and fish nutrients might play a role in the relationship between Hg exposure and neurodevelopment.

APOE is one of the most abundant proteins in the brain. APOE mediates the transportation, redistribution and metabolism of lipids and plays an essential role in neuron repair (Buttini, 1999, Mahley, 1988). One of the biological explanations for the observation of deleterious effects found only in ε4 carriers involves thiol-containing amino acids, which are involved in one of the major pathways of MeHg elimination.

Epsilon 2 and ε3 but ε4 of APOE, contain cysteine, a thiol containing amino acids, in the receptor-binding region. Therefore, ε4 carriers might be at a greater risk of Hg-induced neurological disease development due to the accumulation of Hg in brain tissue (Godfrey, 2003). Additionally, we observed a higher ratio of Hg (maternal blood divided by cord blood) among ε4 carriers compared with ε2 and ε3 carriers in our study (data not shown). This observation may also provide a clue to the gene-environment interaction between *Apoe* gene polymorphisms, prenatal Hg exposure and the resulting impact on neurodevelopment.

An occupational cohort study has found that ε4 carriers were more susceptible to central nervous system effects caused by long-term exposure to lead (Stewart, 2002), which is also a neurotoxin like mercury. Meanwhile, the adverse effects of lead in cord blood on 24-month-old Mental Development Index scores was 4 times greater among ε2 and ε3 carriers compared with ε4 carriers, which suggests that ε4 may confer advantages on neurodevelopment during early life Wright, 2003 #20}. These inconsistent results might be due to the different mechanism pathways; in ε4 carriers, these pathways are associated with neurodegeneration and are deleterious after head injury in adults because neuron repair is less efficient, while it may have a protective effect on brain development by conferring enhanced absorption of cholesterol, which plays an important role during early development (Herz and Beffert, 2000, Ohkubo et al. , 2001,

Oria, 2005, Weisgraber et al. , 1982). In the current study, we found comparable birth outcomes in ε4 carriers at different exposure levels; however, the DQs of CDIIT were significantly lower among the ε4 carriers with higher cord blood Hg levels.

Lead, environmental tobacco smoke (ETS) and breastfeeding are recognized as important confounding factors with the potential to affect neurodevelopment. The primary strength of our study is our use of reliable biomarkers for the assessment of lead and ETS exposure. Measurements of lead and cotinine levels in the cord blood were more reliable indicators of lead and ETS exposure, respectively. The adverse effect of cotinine levels in cord blood on neurodevelopment has been reported in a cohort study (Hsieh, 2008). We found that cord blood lead and cotinine levels were negatively associated with adverse effects on neurodevelopment (data not shown), which association is consistent with current knowledge. Breastfeeding also displayed a protective effect on neurodevelopment in this study, which is consistent with previous study (Chiu et al. , 2011). The frequency and/or quantity of fish consumption during pregnancy and parity were also recognized as potential confounders that may mask the adverse effects of MeHg on neurodevelopment. Adjusting for fish consumption and parity did not alter the magnitude of the model; therefore, due to the small population, these variables were not included in the final model.

The primary limitation of this study is the possibility of obtaining a Type I error due to the multiple testing. The significant results of the gene-environmental interaction between prenatal mercury exposure and *Apoe* genotype regarding child neurodevelopment might occur by chance. However, the subtests of CDIIT neurodevelopmental tests were highly correlated and the results of the different developmental subtests were associated with mercury exposure among ε4 carriers consistently in this study. We believe that this finding is fairly robust and enables us to conclude that gene modification affects the influence of mercury on general neurodevelopment and cognition in children. Another limitation of the study is the low respondent rate. The blood mercury and lead levels of the participants were slightly higher than those of the non-participants, while the maternal characteristics of the participants included older age ranges, higher education levels and higher family income levels compared with those of the non-participants. These variations may promote selection bias. However, a higher socioeconomic status is generally associated with a better environment for raising children (e.g., increased educational resources and less exposure to other environmental toxicants). Therefore, our results may not be overestimated.

In the present study, we lack exposure data on polychlorinated biphenyl (PCB), a recognized neurotoxin that humans can be exposed to via fish consumption; however,

we do not believe that such data would affect our findings. PCB concentrations in the blood of pregnant women in Taiwan are relatively low (Wang et al. , 2004), and no episodes of PCB exposure via fish consumption have been reported in Taiwan. Additionally, in the Faroe Islands, people are exposed to relatively high levels of PCB though fish consumption, thereby suggesting that the adverse effects of Hg exposure is greater than that of PCB exposure (Grandjean et al. , 2012). Furthermore, an unclear effect of PCB on neurodevelopment was also reported in a recent study from Japan (Suzuki et al. , 2010). Although we lack data on PCB exposure, we believe that such data might not skew our results on the association between Hg, *Apoe* gene polymorphisms and neurodevelopment.

In conclusion, prenatal Hg exposure was associated with significant adverse effects on cognition, social and whole neurodevelopment DQs at age 2 years among subjects who have at least one Apoe ε4 allele. In this study, the ε2, ε3 and ε4 alleles of Apoe appeared at frequencies of 9.8%, 82.5% and 7.7%, respectively. The small proportion of ε4 carriers in our study may be the reason that no adverse effects were found without the stratification of the gene. The inter-population difference prevalence of the alleles might be one of explanation for the inconsistent findings related to prenatal Hg exposure and neurodevelopment.

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Table 2- 1 Characteristic of study subjects stratified by cord blood mercury level and three categories of APOE[a] genotypes

	Cord Blood Mercury					
	<12 μg/L			>12 μg/L		
	ε2 carrier	ε3/ε3	ε4 carrier	ε2 carrier	ε3/ε3	ε4 carrier
Total subjects=168	N=17	N= 51	N= 12	N= 15	N= 59	N= 14
Cord Blood Mercury (μg/L, mean±SD)	7.2 ±2.4	8.3 ±2.8	7.3 ±1.9	19.6 ±5.9	20.6 ±9.8	24.0 ±7.3
Child characteristics						
Male (N, %)	9 (52.9)	26 (51.0)	6 (50.0)	9 (60.0)	36 (61.0)	7 (50.0)
Parity 1 (N, %)	7 (41.2)	25 (49.0)	4 (33.3)	5 (33.3)	25 (42.4)	4 (28.6)
Cord Blood Lead (μg/dL, mean±SD)	1.3 ±0.9	1.2 ±0.7	1.3 ±0.7	1.5 ±0.9	1.4 ±0.7	1.4 ±0.6
Cord Blood Cotinine (μg/L, mean±SD)	0.1 ±0.1	0.2 ±0.2	0.2 ±0.2	0.1 ±0.1	0.2 ±0.5	0.1 ±0.03
ETS at 2 years of child's age (N, %)	4 (23.5)	21 (41.2)	6 (50.0)	6 (40.0)	20 (33.9)	6 (42.9)
Maternal characteristics						
Foreigner mother ((N, %)	2 (11.8)	4 (7.8)	1 (8.3)	0 (0)	4 (6.8)	1 (7.1)
Age when delivery (N, %)						
25-35	13 (76.5)	38 (74.5)	11 (91.70	12 (80.0)	35 (59.3)	9 (64.3)
>=35	2 (11.8)	9 (17.6)	1 (8.3)	3 (20.0)	17 (28.8)	5 (35.7)
Maternal Education (N, %)						
High School	9 (52.9)	19 (37.3)	4 (33.3)	7 (46.7)	24 (40.7)	6 (42.9)
College and above	7 (41.2)	29 (56.9)	6 (50.0)	8 (53.3)	34 (57.6)	7 (50.0)
Marine Fish consumption (bowl/week, mean±SD.) *	0.5 ±0.7	0.5 ±0.7	0.4 ±0.3	0.6 ±0.6	1.3 ±1.7	0.9 ±1.0
Family income >1,000,000 at 2 years age (N, %)	4 (23.5)	29 (56.9)	6 (50.0)	10 (66.7)	33 (55.9)	9 (64.3)
HOME[b] score (mean±S.D.)	40.7 ±2.1	41.0 ±2.4	41.9 ±1.9	41.1 ±2.6	40.1 ±3.2	41.2 ±2.9
Ever Breastfeeding (N, %)	14 (82.4)	45 (88.2)	10 (83.3)	11 (73.3)	55 (93.2)	12 (85.7)
Duration of breastfeeding (months, mean±S.D)	4.7 ±7.7	5.0 ±6.0	6.0 ±7.3	6.8 ±7.8	5.2 ±5.6	6.6 ±6.7

[a]APOE, Apolipoprotein E; [b] HOME, Home Observation for Measurement of the Environment; *$p<0.05$* by ANOVA test

Table 2- 2 Birth outcome and CDIIT[a] score at the age of 2 years of study subjects stratified by cord blood mercury level and three categories of APOE[b] genotypes

	Cord Blood Mercury						
	<12 μg/L			>12 μg/L			
	ε2 carrier	ε3/ε3	ε4 carrier	ε2 carrier	ε3/ε3	ε4 carrier	
Total subjects=168	N= 17	N= 51	N= 12	N= 15	N= 59	N= 14	*p-value*[d]
Birth Weight (g, mean±SD)	2934.6 ±504.6	3246.6 ±385.9	3489.6 ±347.4	3129.5 ±486.3	3203.6 ±503.8	3238.9 ±588.5	
Birth Length (cm, mean±SD)	47.8 ±2.3	49.5 ±2.1	49.9 ±1.5	48.7 ±2.2	49.2 ±2.4	48.6 ±1.6	
Head circumference (cm,	33.5 ±1.7	33.9 ±1.5	34.0 ±1.6	33.3 ±1.3	33.7 ±1.8	33.3 ±1.7	
Preterm (N, %)	3 (17.6)	3 (5.9)	0 (0)	1 (6.7)	5 (8.5)	1 (7.1)	
Low birth weight (N, %)	3 (17.3)	2 (3.9)	0 (0)	2 (13.3)	4 (6.8)	1 (7.1)	
Small for gestation age (N, %)	0 (0)	2 (3.9)	0 (0)	1 (6.7)	1 (1.7)	2 (14.3)	
CDIITs[a] Score at 2 year old							
Whole test	100.2 ±10.3	96.9 ±14.6	110.4 ±15.2	98.2 ±12.3	97.3 ±11.8	92.8 ±10.4	<0.05
Cognitive	99.2 ±9.8	90.7 ±14.8	100.8 ±12.9	93.6 ±8.7	92.9 ±12.3	88.7 ±10.4	<0.05
Language	101.1 ±10.9	98.0 ±13.5	107.1 ±13.4	98.8 ±12.1	99.7 ±13.1	96.3 ±9.3	
Gross-motor	76.5 ±16.9	84.3 ±11.0	91.4 ±17.5	82.4 ±11.8	86.7 ±14.6	88.5 ±8.5	<0.05
Fine-motor	102.6 ±12.5	91.5 ±14.7	97.9 ±8.8	100.5 ±13.4	94.3 ±12.2	89.6 ±10.7	<0.01
Motor	86.1 ±13.3	85.2 ±12.4	94.0 ±14.2	87.8 ±12.5	88.2 ±12.7	86.6 ±6.7	
Social	114.4 ±13.2	107.7 ±19.6	122.7 ±17.8	108.1 ±17.4	105.6 ±14.7	102.7 ±14.0	<0.05
Self-help	98.4 ±15.0	102.0 ±18.6	109.1 ±17.4	102.4 ±14.7	100.3 ±17.2	94.1 ±19.2	

[a] CDIITs, Comprehensive Developmental Inventory for Infants and Toddlers; [b] APOE, Apolipoprotein E; [c] DQs, developmental quotients.
[d] *p* value of ANOVA test

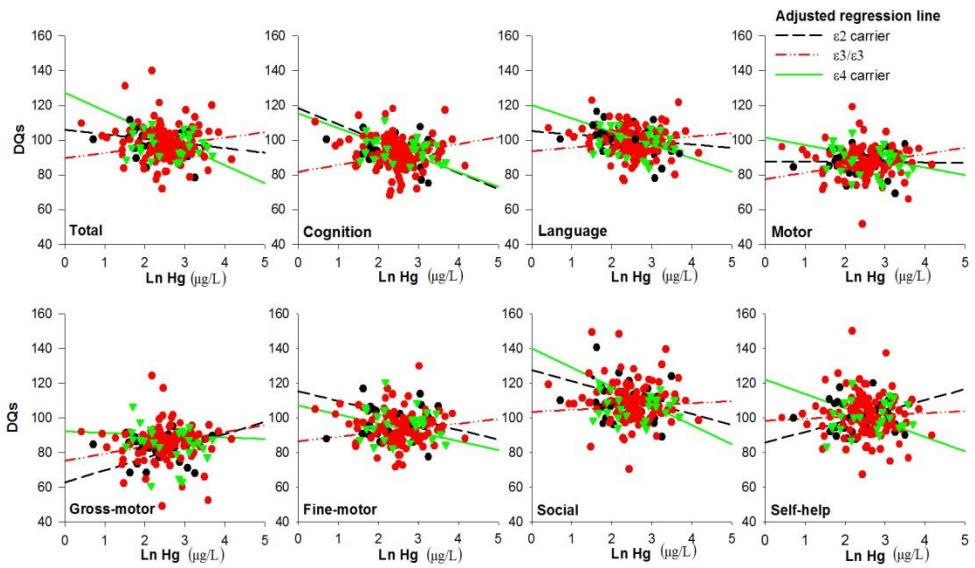
Table 2- 3 Multiple linear regression of neurodevelopment at age of 2 years by blood mercury and APOE[a] gene polymorphisms.

			-coefficient of mercury concentration [ln (ng/mL)] in CDIITs[b] ,DQs[c]							
Model	Subjests	N	Whole test	Cognitive	Language	Gross-motor	Fine-motor	Motor	Social	Self-help
Crude										
Model 1	All subjects	168	-1.2	0.04	-0.05	3.14	0.11	1.73	-1.96	-3.50
			(-4.45, 2.04)	(-3.12, 3.19)	(-3.22, 3.11)	(-0.14, 6.43)	(-3.08, 3.29)	(-1.34, 4.79)	(-6.16 ,2.24)	(-7.81 ,0.80)
	2 carrier	32	0.08	-5.28	-0.86	6.68	-0.9	3.08	-0.64	-4.43
			(-6.60, 6.75)	(-10.66, 0.10)	(-7.62, 5.91)	(-1.81, 15.17)	(-8.49, 6.70)	(-4.43, 10.59)	(-9.81 ,8.53)	(-22.49 ,-0.87)
Model 2	3/ 3	110	1.37	3.59	1.91	3.47	2.24	3.37	0.21	-2.95
			(-2.68, 5.42)	(-0.54, 7.72)	(-2.18, 6.00)	(-0.53, 7.46)	(-1.90, 6.38)	(-0.49, 7.23)	(-5.10 ,5.51)	(-8.44 ,2.54)
	4 carrier	26	**-12.40****	-7.53	-6.62	-2.02	**-6.88***	-6.02	**-11.68***	**-14.44***
			(-20.79, -4.02)	(-15.18, 0.12)	(-14.04, 0.79)	(-10.43, 6.39)	(-12.97, -0.79)	(-12.73, 0.69)	(-22.49 ,-0.87)	(-25.37 ,-3.51)
Interaction p	All subjects	168	**0.0045**	**0.0034**	0.0528	0.3981	**0.0386**	**0.0463**	0.0621	0.1875
Adjusted										
Model 3[g]	All subjects	168	-0.04	0.02	-0.29	**3.95***	0.72	2.26	-1.79	0.1
			(-3.21, 3.12)	(-3.18, 3.23)	(-3.44, 2.86)	(0.51, 7.39)	(-2.47, 3.91)	(-0.93 ,5.45)	(-6.18 ,2.60)	(-4.15 ,4.36)
	2 carrier	32	-2.66	**-9.31*****	-1.93	6.97	-5.59	-0.16	-6.32	6.13
			(-10.18, 4.85)	(-14.75, -3.88)	(-10.25, 6.39)	(-2.25, 16.19)	(-13.14, 1.96)	(-8.10 ,7.79)	(-18.74 ,6.11)	(-3.14 ,15.40)
Model 4[h]	3/ 3	110	2.98	4.03	2.08	4.11	2.51	3.61	1.22	1.08
			(-0.99, 6.95)	(-0.13, 8.19)	(-2.03, 6.18)	(-0.14, 8.35)	(-1.68 , 6.69)	(-0.47 ,7.69)	(-4.38 ,6.83)	(-4.71 ,6.88)
	4 carrier	26	**-10.45****	**-8.47***	-7.64	-0.87	-5.15	-4.35	**-11.02***	-8.30
			(-17.36, -3.54)	(-16.10, -0.84)	(-15.58, 0.29)	(-9.22, 7.48)	(-11.72, 1.42)	(-10.68 ,1.98)	(-20.85 ,-1.19)	(-18.70 ,2.09)
Interaction p	All subjects	168	**0.0007**	**0.0006**	**0.0194**	0.5045	**0.0357**	0.0646	0.0504	0.0863

[a] APOE, Apolipoprotein E; [b] CDIITs, Comprehensive Developmental Inventory for Infants and Toddlers; [c] DQs, developmental quotients

[d] Model 1: Y= B0+B1(CB Hg) + B2(2 carrier(yes or no)) +B3(4 carrier (yes or no))+e; [e] Model 2: Subjects stratified by 3 categories of APOE genotype: ε2 carrier, ε3/ε3 and ε4 carrier, and then estimated the effect of CB Hg on neurodevelopment, Y= B0+B1(CB Hg) +e; [f] p values of interaction term (APOE*CB Hg) estimated in the model: models 1 + an interaction term (APOE*cord blood Hg)]; [g] Model 3: Model 1 + covariates: infant sex, birth weight, maternal nationality, maternal educational level, maternal age at delivery, HOME score, family income at aged 2 years, concentrations of lead and cotinine in cord blood, and duration of breastfeeding. [h] Model 4: Model 2 + covariates: infant sex, birth weight, maternal nationality, maternal educational level, maternal age at delivery, HOME score, family income at aged 2 years, concentrations of lead and cotinine in cord blood, and duration of breastfeeding; [i] p value of interaction term (APOE*cord blood Hg) estimated in the model: model 3 + an interaction term (APOE*cord blood Hg).

p<0.05; **p<0.01; ***p<0.005



● ε2/ε2 and ε2/ε3

● ε3/ε3

▼ ε3/ε4

Figure 2- 1 Stratified [a] multiple linear regressions of each Developmental Quotients (DQs) of CDIIT test and cord blood Hg levels.

[a] Subjects stratified by *Apoe* genotype

Table 2S1 Multiple linear regression of neurodevelopment at age of 2 years by blood mercury and APOE[a] gene polymorphisms

	Included		Excluded	
	N	%	N	%
Infant characteristics				
Male	93	55.4	154	48.4
Birth Order				
1	70	41.7	160	50.3
>=2	98	58.3	158	49.7
Birth Outcome				
Preterm (%)	13	7.7	29	9.1
Low birth weight	12	7.1	26	8.2
Small for gestation age	6	3.6	28	8.8
Maternal Characteristic				
Foreign mother (%)	12	7.1	28	8.8
Age (%)				
<25	13	7.7	41	12.9
25-35	118	70.2	216	67.9
>=35	37	22.0	61	19.2
Education Level				
Junior high school	8	4.8	37	11.6
High School	69	41.1	157	49.4
College and above	91	54.2	124	39.0
Self-report active smoker	0		8	2.5
Environmental Tobacco Smoke (%)				
Yes	42	25.3	96	30.4
Consume Fish during pregnancy (%)	163	97.0	293	92.4
Annual family income >=1,000,000 (NTD)	82	50.1	112	36.0

Chapter Three: Mercury, APOE, and child behavior

3.1 Introduction

Methylmercury (MeHg) is able to penetrate the blood-brain and placental barriers and may adversely affect reproduction and neurodevelopment (Clarkson and Magos, 2006, Farina, 2011, Yee and Choi, 1996). Because one of its features is bioaccumulation, high concentrations of MeHg can accumulate in large predator fish (Clarkson, 2002, Clarkson and Magos, 2006). Animal studies designed to mimic human gestational MeHg exposure found a significant decrease in motor and mnemonic capabilities in exposed mice as well as a minor increase in attention deficit and anxiety concentrations (Liang, 2009, Montgomery, 2008). Adverse impact of prenatally exposure to MeHg through fish consumption on children's cognition (Grandjean, 1997) (Grandjean, 1998) (Lederman, 2008) and behavior (Boucher, 2012, Sagiv, 2012) (Debes, 2006) has been reported in studies at different degrees of prenatal MeHg exposure at different countries. However, in Seychelles cohort studies which their study subjects prenatally exposed to a moderate level of MeHg (approximately 6 μg/g in maternal hair), no association between prenatal MeHg exposure and children cognition and problematic behavior has been reported (Davidson, 1998, Davidson, 2006b, Myers, 2003, Myers, 2000). One of the primary explanations of these inconsistencies is the compensatory effect of nutrients

in seafood that overcome the neurotoxicity of MeHg (Sakamoto, 2004, Stokes-Riner, 2011, Strain, 2008).

In addition to the hypothesis that the nutrients in fish can mitigate or overcome the negative effects of MeHg exposure, it is possible that there is a genetic susceptibility to MeHg toxicity. An increasing amount of evidence has demonstrated that genetic susceptibility may modify the effect of prenatal Hg exposure on the development of children. A Korean birth cohort study indicated that maternal glutathione S-transferase M1 and glutathione S-transferase T1 polymorphisms modified the effect of Hg toxicity on infant birth weight (Lee, 2010). In a previous study, we determined that individuals with the Apolipoprotein E (*APOE,* gene; Apoe, protein) epsilon 4 (ε4) variant were more susceptible to MeHg toxicity, and this variant was associated with poorer neurodevelopment of children who were 2 years of age (Ng et al. , 2013). Recently, the Avon Longitudinal Study of Parents and Children cohort study found several gene polymorphisms, including one in Apolipoprotein A (*APOA*), that might modify the impact of Hg on intelligent quotients in children (Julvez et al. , 2013).

Apoe is recognized as a crucial factor involved in cholesterol metabolism, neurite growth and neuron repair in the central nervous system (Buttini, 1999, Mahley, 1988). *APOE* has three common variants: epsilon2 (ε2), epsilon3 (ε3) and epsilon4 (ε4) (Zannis, 1982). *APOE* ε4 has been recognized as a risk factor for Alzheimer's disease

(Kim et al. , 2009). A previous longitudinal study suggested that an *APOE* ε4 carrier might be associated with hyperactivity, emotional reactions and sociability problems in children and young adults (Keltikangas-Jarvinen, 1993). However, several studies reported that the ε4 variant was associated with a neurodevelopmental advantage in children that had an unfavorable exposure or nutrient deficiency (Oria, 2005, Wright, 2003). Whether carriers of the *APOE* ε4 variant are more susceptible to neurological disorders or whether this variant confers an advantage against unfavorable exposures remains unclear.

To the best of our knowledge, no study has investigated the effect of *APOE* genetic susceptibility and prenatal exposure to Hg on child behavior. Therefore, the aim of this study was to investigate the role of *APOE* gene polymorphisms and the relationship between cord blood Hg concentrations and child behavior in two-year-old children.

3.2 Material and methods

Study designs and subject recruitment

This is a study from the Taiwan Birth Panel study (TBPS). The TBPS is a prospective cohort study conducted between April 2004 and January 2005 at a medical center, a local hospital and two clinics in northern Taiwan. Four hundred and eighty-six mother-infant pairs participated in the TBPS. The participants' guardians were informed of the research purpose and research procedures. With their permission, the cord blood of each participant was collected at delivery by a standard protocol. Participants' information, including family demographics, home characteristics, lifestyle factors and family medical history, were collected before the participants were discharged from the hospital (Hsieh, 2011).

Two hundred and sixty-six parents from the TBPS cohort responded to the child behavior evaluation when their children were two years old. Informed consent was obtained again before additional data were collected, including data regarding child behavior and the parenting environment. All of the protocols were approved by the Ethical Committee of the National Taiwan University Hospital. The study subjects were restricted to those with cord blood Hg (450 subjects), *APOE* genotype (347 subjects) and neurodevelopment test data (266 subjects). A total of 172 subjects were eligible. Among these 172 subjects, 3 subjects with an ε2/ε4 *APOE* genotype, 1 subject with a

genetic disease, 1 subject with cord blood cotinine concentrations greater than 15 μg/L (Peacock et al. , 1998) and 1 subject whose mother self-reported as "used to be an active smoker" were excluded. Overall, 166 subjects were included in this study.

Measurement of prenatal mercury (Hg) exposure

The concentration of total Hg was measured in the cord blood for determining Hg exposure status. Whole blood was collected in ethylene-diamine-tetraacetic acid (EDTA) tubes and stored at -80 ℃ until the laboratory analysis was carried out. The analysis of 18 metals in the blood, including Hg, was performed on an inductively coupled plasma mass spectrometer (ICP-MS, 7500C, Agilent Technologies, Japan) (Barany, 1997). For quality assurance and quality control, 1 spike per 10 samples was used to confirm that the measurements were reliable. The detection limit of Hg in the present study was 0.18 μg/L. The cord blood Hg concentrations of all participants were above the detection limits.

Child Behavior Checklist 1.5/5

We used the Child Behavior Checklist 1.5/5 (CBCL/1.5-5), Chinese version, to measure the behavior problems of the participants at two years of age. The CBCL/1.5-5, which was designed to collect child behavior information from the previous 2 months, is a reliable instrument commonly used to measure children's behavior, emotion and social function at ages from 1.5 to 5 years (Achenbach TM, 2001). The CBCL uses a 3-point

scale (0 for not true, 1 for somewhat or sometimes true, and 2 for very true or often true). It consists of 100 items, 99 of which assess specific behavior problems and 1 open question for parents to list any problems that are not included in the checklist. Of the 100 items, 67 are divided into 7 narrow-band behavior syndromes, including emotionally reactive, anxious/depressed, somatic complaints, withdrawn, sleep problems, attention problems and aggressive behavior. Of these 7 narrow-band syndromes, 6 are categorized into 2 broad-band syndromes; internalizing problems include the former 4 syndromes, while externalizing problems include the latter 2 syndromes. The reliability and the validity of the CBCL1.5/5 were examined in Taiwan. The internal consistency of the CBCL1.5/5, the Cronbach's alpha coefficient, was 0.62 to 0.95, and the intra-class correlation coefficient (ICC) of test-retest reliability for the syndromes was 0.52 to 0.84. In addition, the inter-parent agreement for the CBCL1.5/5 was acceptable; the ICCs ranged from 0.4 to 0.84 for most of the syndromes (except sleep problems) and the total problem (Wu et al. , 2012).

APOE Genotyping

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to determine the *APOE* genotype. First, Chemagic deoxyribonucleic acid (DNA) specialized for blood kits (Chemagen, Aachen, Germany) was used to extract DNA from the blood. Then, primer pairs--

F5'-TCCAAGGAGCTGCAGGCGGCGCA and

R5'-GCCCCGGCCTGGTACACTGCCA--were used to amplify a 218 base-pair DNA fragment that contained *APOE* polymorphic sites (Zivelin, Rosenberg et al. 1997) in an ABI GeneAmp™ 2700 system. The PCR protocol was initiated with 10 minutes at 95 ℃ for DNA denaturation, followed by 35 thermal cycles: denaturing (94 ℃ for 30 seconds); annealing (65 °C for 45 seconds); extension (72 °C for 45 seconds); and 10 minutes at 72 °C for the last extension. Then, 10 μL of amplified DNA was digested with two restriction enzymes – AflIII (2 units) and HaeII (6 units) – for 24 hours at 37 ℃. After digestion, the products were analyzed in a 4% agarose gel with ethidium bromide staining (Zivelin, 1997).

Covariates

The risk factors that could interfere with the relationship between prenatal Hg exposure and child behavioral problems were considered. The categorical and continuous covariates were controlled in the present study, including: sex, birth order (1^{st}, 2^{nd} and above), maternal age (less than 25, 25 to 35, older than 35 years old), maternal education level (junior high school and below, high school, college and above), family income (less than1,000,000 New Taiwan dollars, equal to or more than 1,000,000 New Taiwan dollars), maternal pregnancy marine fish consumption (continuous), and concentrations of cotinine (continuous), lead (continuous), selenium (continuous) in

cord blood, birth weight (continuous). This information was collected via a structured questionnaire. In addition, the quality and quantity of the caregiving environment, which is recognized as an important factor that may influence child behavior and development, were evaluated via the Home Observation for Measurement of the Environment-Infant/Toddler version (HOME), which was administered by well-trained interviewers when the participant was 2 years of age.

Statistical analysis

The metal and cotinine concentrations observed in the cord blood, CBCL scores and HOME scores for the TBPS cohort and present study were demonstrated in table 3-1, by the values of mean, median and range. The participants were categorized into two groups according to the median concentration of Hg (12 μg/L). The mean and the proportional difference of cord blood Hg in each category of variables were tested by independent T-tests, the analysis of variance (ANOVA) test and the Chi-square test, as shown in table 3-2 and table 3- 3. The independent T-tests and ANOVA were used to compare the mean concentrations of Hg in each categorical variable. The Chi-square test was used to test the proportional difference of the categorical variables. Then, the main effects of Hg and the *APOE* genotype (non-ε4 or ε4 carriers) on child behavior were examined using multiple linear regression models, shown in table 3-4. The dummy variables were applied to represent the combined effect of Hg and *APOE*, in which the

non-ε4 carrier with lower Hg was assumed as the referent; and the combined effects of Hg and the *APOE* genotype on child behavior were calculated using multiple linear regression models, as shown in table 3-5. The mean and standard deviation of CBCL score between sexes was shown in Table 3S- 4. Furthermore, the gender difference in susceptibility to MeHg on child behavior was tested in multiple linear regression models, as shown in Table 3S- 5. Two tailed p-values < 0.05 were considered statistically significant. All analyses were conducted using SPSS 16.0, SAS Version 9.2 and SigmaPlot 10.0.

3.3 Results

One-hundred and sixty-six subjects from the TBPS cohort met our inclusion criteria. The frequencies of the *APOE* genotypes conform to Hardy-Weinberg equilibrium (data not shown). The cord blood Hg of the participants was slightly higher compared with the TBPS cohort. However, the participants' characteristics, including birth outcomes, maternal characteristics and maternal exposure during pregnancy, were homogeneous with the TBPS cohort *(Table 3S-1, 2, and 3).*

Table 3-1 shows the metal and cotinine concentrations observed in the cord blood and the CBCL and HOME scores for the TBPS cohort and present study. The distribution of Hg concentrations and child behavior scores of the TBPS cohort and present study were similar. The median Hg levels of the participants in the present study were 12 μg/L, two times higher than the reference dose recommended by the Environmental Protection Agency of the United States (USEPA). The relationship between the Hg concentrations and demographic characteristics of the participants were examined as both continuous and dichotomous variables. The subjects were stratified into two groups defined by the 12 μg Hg/L threshold. Then, the proportional distributions of subjects and their maternal characteristics between participants with blood Hg less than 12 μg Hg/L (referent Hg group) and participants with blood Hg equal to or higher than 12 μg Hg/L (elevated Hg group) were compared, as shown in table 3-2 and table 3-3. Table 3-2

shows that no association was found between the cord blood Hg level and the children's characteristics. The subjects' and their maternal characteristics were homogenous.

In table 3, the results show that 97% of the participants in the present study ate fish and that the amount of maternal marine fish consumption was responsible for the increased Hg levels in the cord blood in both the referent group and the elevated Hg group. The amount of weekly marine fish consumption was statistically higher in the elevated Hg group (Chi square test, $\chi^2 = 13.79$, *p-value* < 0.01). In addition, cord blood Hg concentrations were positively associated with maternal age and household income, but the proportional distribution between the two stratified groups was homogenous. Furthermore, no significant difference was observed between these two groups for other maternal characteristics, including nationality, age at delivery, education level, and pregnancy exposure to environmental tobacco smoke (ETS).

The crude and adjusted beta coefficients between the Hg concentrations and the *APOE* genotype effect on the CBCL score are shown in table 3-4. Non-ε4 carrier participants with an Hg concentration < 12 μg/L were defined as the referent group in the models. The adjusted models included the following potential confounding factors: participants' characteristics (sex, birth order, and birth weight); other exposures determined by the cord blood (cotinine, lead, cord and selenium); maternal characteristics (age and education); amounts of pregnancy fish-eating; household income; and HOME scores.

The ε4 carrier was associated with higher scores in most of the CBCL syndromes and had a significantly higher score in emotional reaction. After adjusting for potential confounders, the relationship between ε4 carrier status and the syndrome scores remained consistent; being an ε4 carrier was associated with higher scores in all of the syndromes and higher total scores. Although elevated Hg concentrations were not associated with the CBCL score before or after adjustment for the potential confounders, increased cord blood Hg concentrations were positively associated with all of the syndromes, except the emotionally reactive syndrome.

In Table 3S-5 and figure 3-1, the combined effects of Hg and *APOE* on child behavior were calculated, and the non-ε4 carrier with a lower Hg concentration was defined as the referent. Compared with the referent, ε4 carriers with elevated cord blood Hg concentrations had an increased score for all of the syndromes tested after adjusting for the confounding factor. Total CBCL score (ß ± SE =14.3 ± 6.1, $p < 0.05$), total internalizing score (ß ± SE = 5.6 ± 2.1, $p < 0.05$), emotionally reactive (ß ± SE = 1.8 ± 0.7, $p < 0.05$) and anxious/depressed (ß ± SE = 2.1 ± 0.7, $p < 0.01$) were statistically significantly higher in the ε4 carrier with an elevated Hg concentration. In addition, the beta coefficients of externalizing and aggressive syndromes were of borderline significance. Furthermore, the gender difference susceptibility to MeHg on child's behavior was shown in Table 3S-4 and Table 3S-5. In present study, boys tended to

have higher score in CBCL than girls. Girl subjects with the *APOE* ε4 allele were found to have higher score in be associated with an increased score in anxious/depression symptoms (Table 3S-4). After adjusting for MeHg exposure, the association was even greater (Table 3S-5). In summary, ε4 carriers with elevated Hg levels tended to have higher CBCL scores.

3.4 Discussions

In this study, elevated Hg concentrations in ε4 carriers were associated with a higher score (higher score represents poorer performance on the CBCL) in the general behavior test and in all CBCL syndromes compared to the other *APOE* variants, although no association was found between Hg levels and child behavior. The ε4 carriers had significantly higher scores in general internalizing behaviors and its sub-class syndromes: emotionally reactive and anxious/depressed. Besides, they also had borderline significantly higher scores in general externalizing and aggressive syndromes. No gender difference in susceptibility to MeHg was found until the data were adjusted for *APOE* variants. However, because the sample size was small, we hesitate to draw any conclusions regarding gender differences in susceptibility to MeHg or *APOE* on child behavior. Thus, our results suggest that ε4 carriers may be more vulnerable to MeHg and that elevated Hg concentrations most likely increase the susceptibility of behavior problems in *APOE* ε4 carriers.

Although the median Hg concentration in our study subjects was 12 μg/L, approximately two times higher than the reference dose recommended by the USEPA, no association was found until *APOE* genetic susceptibility was considered. Two cohort studies in North America found that Hg concentrations were associated with increased inattention and aggressive behavior in school children (Boucher, 2012, Sagiv, 2012),

while no association was found in the Seychelles cohort study, even though the Hg exposure concentrations in the Seychelles were higher (Myers, 2000). These inconsistencies could be explained primarily by the nutrients provided through fish consumption. However, a fish-eating diet is not the only explanation and may be inadequate to explain all incompatible findings. Additionally, it is rational to assume that the genetic polymorphisms regulating fatty acids and cholesterol may modify the effect of Hg on child neurodevelopment because cholesterol and fatty acid are critical for the development of the brain. A similar finding, that the minor *APOA* variant may be associated with a cognitive deficit in 8-year-old children, has been reported from the Avon cohort study. Overall, our findings suggest that *APOE* genetic variants may alter the effect of Hg by providing an example of how genetic susceptibilities may modify the toxic effects of MeHg.

Different *APOE* alleles may vary the susceptibility of an individual to MeHg-related neurological disorders. Cholesterol and fatty acids are essential for brain development. The ε4 allele is associated with elevated cholesterol absorption in the intestine and may be potentially advantageous in early neurodevelopment (Oria, 2005, Weisgraber, 1982). However, the ε2 and ε3 alleles contain at least one cysteine, an amino acid that contains a thiol functional group, which mediates the elimination of MeHg, at positions 112 and 158, while the ε4 allele does not have a cysteine at either site that might be associated

with increased Hg levels. Furthermore, the ε4 allele was also found to have limited neuron repair ability (Buttini, 1999, Herz and Beffert, 2000, Mahley, 1988, Ohkubo, 2001). Finally, it has been suggested that the ε4 allele may be associated with a higher risk of suffering from neurological diseases and an increased susceptibility to Alzheimer's disease (Godfrey, 2003). When MeHg crosses the blood-brain barrier and causes damage in the brain, ε4 carriers may perform less efficiently in the elimination of MeHg and neuron repair compared to non ε4 carriers.

Previous studies suggested that the sexes may vary in their susceptibility to MeHg toxicity. However, a recent review concluded that there is an unclear pattern in gender susceptibility to MeHg toxicity (Llop et al. , 2013). One animal Hg toxicokinetic study found that both sex and strain modified the accumulation of Hg. They also found that hybrid offspring (F2) had a larger variance in the retention of Hg. Different strains of mice may vary by gender in susceptibility to Hg (Ekstrand et al. , 2010). In this study, we observed a tendency for boys to have higher CBCL scores than girls. After adjusting for the *APOE* variants, we observed a positive association between Hg and the CBCL score and a tendency for girls who were ε4 carriers with elevated Hg levels to have more anxious/depression symptoms than boys. Therefore, we can infer that the differences in genetic predisposition may be one of the possible reasons for the inconsistencies in gender susceptibility.

A North American study suggested that prenatal Hg exposure was associated with externalizing behavior, including inattention and aggression (Boucher, 2012, Sagiv, 2012); however, our results demonstrated that ε4 carriers tend to have significantly higher scores in internalizing syndromes (table 3-4) rather than externalizing syndromes. These inconsistencies may be due to two main reasons. First, the participants' ages were different in each study; the subjects in the Canadian and United States studies were school children, while the participants in this study were 2 years old. Current knowledge suggests that externalizing syndromes are easier to evaluate in lower grade school children, making it difficult to compare the findings between 2-year-old and 8-year-old or older children. The second reason was cultural differences. A previous study indicated that the high control and authoritarian parenting style may be responsible for the tendency of Taiwanese preschoolers to have higher prevalence rates in internalizing syndromes than children from Western countries (Wu, 2012). In animal studies mimicking human exposure, exposed mice performed with more inattention and/or anxious-like behavior in addition to deficit neurodevelopment (Liang, 2009, Montgomery, 2008). However, it is difficult to project the results of animal behavior studies to humans. The current knowledge on the biological plausibility that *APOE* may mediate internalizing behavior is limited. Therefore, we hesitate to draw any conclusion on these differences due to the inconclusive information.

The primary limitation of our study was the possibility of obtaining a type I error due to the multiple testing. However, the relationship between each CBCL subtest, Hg and *APOE* were coherent. Because these CBCL subtests are highly correlated, these consistent findings support the conclusion that our results were fairly robust. Another limitation was the low response rate, and the demographic variables between respondents and non-respondents may lead to a selection bias. The differences include slightly higher blood Hg and Pb levels, older maternal age range and higher education level and household income in participants. However, a better socioeconomic level is correlated with providing better resources for the child. Thus, our results might not be overestimated. Last, although polychlorinated biphenyl (PCB), a neurotoxin that humans may be exposed to via fish consumption, was not investigated, the adverse effect of Hg was greater than that of PCB, as reported in a Faroese study (Grandjean et al. , 2012). Furthermore, the blood PCB concentration in Taiwanese pregnant women is relatively low (Wang et al. , 2004), and there were no reports of high PCB exposure in Taiwan from fish. Therefore, the claim that the lack of the exposure information for PCB may interfere with our finding was limited.

Although some limitations were present, this study was able to adequately provide scientific information regarding the effect of Hg on child behavior and the modifying role of *APOE* for the following reasons. We used a reliable biomarker for prenatal Hg

exposure (Grandjean et al. , 2005) and valid questions for the child behavior measurements. Additionally, the information on the potential confounders was accurate and comprehensive because the exposure levels of cotinine, Pb and selenium were directly measured in cord blood and the parenting environment was evaluated by a home visit. The questionnaires that provided information about social demographic characteristics and medical history were administered twice, once at birth and when the subjects were two years old, and the exposure status and outcome were blinded to the parents. These advantages minimized the possibility of recall bias and misclassification. Furthermore, our finding was in accordance with the common knowledge that boys had higher behavior scores and the amounts of maternal fish consumption and blood selenium were inversely associated with the behavior score. Therefore, our results support the conclusion that an individual's *APOE* genetic polymorphisms may modify susceptibility to the toxic effects of Hg.

There was consistency in the results of this study: subjects who were ε4 carriers with elevated cord blood Hg levels had increased scores in all CBCL syndromes compared with the reference group. Our results showed that the elevated Hg in cord blood most likely enhances the risk of deficit behavior in preschool children who are *APOE* ε4 carriers. This finding also supports our previous finding that *APOE* ε4 may modify the relationship between Hg and child neurodevelopment (Ng, 2013). We may infer that

different genetic polymorphisms of *APOE* may modify the effect of MeHg on child health. Thus, we suggest that genetic susceptibility should be considered in future studies that investigate the toxicity of Hg exposure on children's health and that this may help to classify the impact of MeHg on child health. Biological mechanistic studies are needed to clarify the direct or indirect modifying effect of *APOE* variants on MeHg-induced neurotoxicity.

3.5 Reference

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Table 3- 1 Comparison of cord blood metals, cotinine and child behavior scores between the TBPS cohort and present study

	TBPS cohort						Present study				
Exposure Characteristics	N	Mean	SD	Median	Min	Max	N	Mean	SD	Median	Min	Max
Cord Blood Hg (μg/l)	450	14.1	9.1	11.8	1.3	70.8	166	14.7	8.7	12.0	1.53	47.1
Cord Blood Pb (μg/l)	429	1.3	0.73	1.1	0.02	5.2	158	1.3	0.7	1.1	0.05	3.5
Cord Blood Se (μg/l)	450	197.3	45.9	199.3	57.3	349.9	166	203.7	41.0	205.6	97.0	312.0
Cord Blood Cotinine (μg/l)	319	3.5	19.4	0.13	0.006	205.3	99	0.2	0.4	0.1	0.006	3.9
Child Behavior Checklists												
Total problems	266	45.8	20.3	45	6	109	166	46.6	21.3	46	6	109
Internalizing	266	11.9	7.3	11	0	32	166	12.0	7.3	10	0	32
Externalizing	266	15.6	7.4	16	0	39	166	15.8	7.7	16	0	39
Narrow-band syndromes												
Emotionally reactive	266	3.0	2.4	2	0	11	166	3.1	2.5	3	0	11
Anxious/depressed	266	3.6	2.3	3	0	12	166	3.7	2.3	2	0	12
Somatic complaints	266	2.9	2.3	2	0	10	166	2.8	2.2	2	0	9
Withdrawn	266	2.3	1.9	2	0	9	166	2.3	2.0	3	0	9
Attention problems	266	2.9	1.8	3	0	9	166	3.0	1.9	3	0	9
Aggressive behavior	266	12.7	6.2	12	0	32	166	12.8	6.4	13	0	32
Sleep problems	266	4.3	2.3	4	0	12	166	4.4	2.4	4	0	12
HOME Score	273	40.5	3.1	41	29	45	148	40.7	3.0	41	30	45

Abbreviation: TBPS, Taiwan birth panel study; HOME, Home Observation for Measurement of the Environment

Table 3- 2 Child the characteristics and birth outcomes of participants in present study

	N (%)	Cord Blood Hg (μg/L)		Cord Blood Hg (μg/L)		
		Mean (SD)	*p*	<12 N=80	>=12 N=86	*p*
Sex						0.38
Male	95 (57.2)	15.2 (8.7)	0.39	43	52	
Female	71 (42.7)	14.0 (8.8)		37	34	
Birth Order						0.18
1	72 (43.4)	13.8 (8.3)	0.27	39	33	
>=2	94 (56.6)	15.3 (9.0)		41	53	
APOE genotype						0.87
ε2 carrier	28 (16.9)	14.0 (7.6)	0.47	13	15	
ε3/ε3	111 (66.9)	14.4 (8.7)		55	56	
ε4 carrier	27 (16.3)	16.5 (10.1)		12	15	
Preterm (%)						0.46
Yes	13 (7.8)	12.9 (5.9)	0.45	5	8	
No	153 (92.2)	14.8 (8.9)		75	78	
Low birth weight						0.54
Yes	11 (6.6)	13.8 (7.0)		4	7	
No	155 (93.4)	14.7 (8.9)	0.72	76	79	
SGA (%)						0.46
Yes	6 (3.6)	18.8 (11.1)	0.24	2	4	
No	160 (96.4)	14.5 (8.6)		78	82	

Abbreviation: ETS, environmental tobacco smoke; SGA, small for gestational age

Table 3- 3 Social demographic and maternal pregnancy exposure characteristics in the present study

	N (%)	Cord Blood Hg (μg/L)		Cord Blood Hg (μg/L)		
				<12	>=12	
		Mean (SD)	*p*	N=80	N=86	*p*
Age (%)			0.03			0.13
< 25	10 (6.0)	11.8 (6.5)		4	6	
25 - 35	121 (72.9)	13.9 (8.4)		64	57	
>= 35	35 (21.1)	18.0 (9.8)		12	23	
Maternal Education			0.16			0.54
Junior high school	9 (5.4)	9.6 (5.4)		6	3	
High school	68 (41.0)	14.4 (8.4)		33	35	
College and higher	89 (53.6)	15.4 (9.1)		41	48	
ETS during pregnancy			0.32			0.29
No	123 (74.1)	14.2 (8.6)		62	61	
Yes	43 (25.9)	15.8 (9.1)		18	25	
Fish Consumption [a]			0.30			0.20
No	5 (3.0)	10.7 (10.3)		4	1	
Yes	161 (97.0)	14.8 (8.7)		76	85	
Marine Fish (ounces/week)			0.03[c]			<0.01
0	10 (6.0)	11.5 (10.5)		8	2	
< 1	43 (25.9)	14.3 (8.5)		22	21	
>= 1 & < 2	32 (19.3)	13.0 (7.7)		18	14	
>= 2 and < 7	55 (33.1)	14.6 (8.9)		27	28	
>= 7	26 (15.7)	18.7 (8.6)		5	21	
Annual family income [b]						0.15
< NT 1,000,000	70 (42.2)	13.1 (7.5)	0.03	37	33	
>= NT 1,000,000	96 (57.8)	16.2 (9.3)		43	53	

Abbreviation: ETS, environmental tobacco smoke; NT, New Taiwan Dollar

[a] Fish consumption includes freshwater fish and marine fish

[b] Annual family income when participants were 2 years old

[c] *p* for trends

Table 3- 4 Simple and multiple linear regression models of CBCL by the cord blood mercury level and *APOE* genotype

	Cord blood Hg levels <12 ug/L vs. >=12 ug/L				*APOE* genotype non 4 carrier vs. 4 carrier			
	Crude [a]		Adjusted [a,b]		Crude [c]		Adjusted [c,d]	
Child Behavior Checklists	ß (SE)	*p*	ß (SE)	*p*	ß (SE)	*p*	ß (SE)	*p*
Total problems	0.004 (3.313)	1.00	2.331 (3.488)	0.50	5.901 (4.462)	0.19	9.823 (4.369)	<0.05
Internalizing	0.003 (1.143)	1.00	0.624 (1.224)	0.61	2.269 (1.537)	0.14	3.539 (1.530)	<0.05
Externalizing	-0.009 (1.207)	0.99	0.714 (1.243)	0.57	1.395 (1.630)	0.39	2.690 (1.567)	0.09
Narrow-band syndromes								
Emotionally reactive	-0.235 (0.394)	0.55	-0.120 (0.428)	0.78	1.056 (0.528)	<0.05	1.442 (0.531)	<0.01
Anxious/depressed	0.200 (0.360)	0.58	0.477 (0.388)	0.22	0.884 (0.483)	0.07	1.242 (0.486)	<0.05
Somatic complaints	-0.008 (0.337)	0.98	0.125 (0.366)	0.73	0.436 (0.456)	0.34	0.647 (0.462)	0.16
Withdrawn	0.047 (0.312)	0.88	0.142 (0.336)	0.67	-0.108 (0.422)	0.80	0.208 (0.427)	0.63
Attention problems	-0.021 (0.290)	0.94	0.173 (0.305)	0.57	0.146 (0.393)	0.71	0.498 (0.386)	0.20
Aggressive behavior	0.011 (1.004)	0.99	0.541 (1.039)	0.60	1.249 (1.356)	0.36	2.192 (1.310)	0.10
Sleep problems	0.152 (0.370)	0.68	0.386 (0.394)	0.33	-0.040 (0.501)	0.94	0.235 (0.502)	0.64

Abbreviation: ß, Beta-coefficient; SE, standard error

[a] Beta-coefficient of cord blood mercury (reference <12 ug/L);

[b]Model adjusted for participants' sex, birth order, cotinine, lead and selenium levels in cord blood, birth weight, maternal age, maternal education level, maternal pregnancy marine fish consumption, HOME and family income.

[c] Beta-coefficient of the APOE genotype (reference non 4 carrier);

[d]Model adjusted for participants' sex, birth order, cotinine, lead and selenium levels in cord blood, HOME, birth weight, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption.

Table 3- 5 Multiple liner regression of the CBCL score of participants at age 2 by APOE genotypes and cord blood Hg levels

		Non ε4 carrier		ε4 carrier			
	<12 ug/l	>= 12 ug/L		< 12 ug/L		>= 12 ug/L	
Child Behavior Checklists		ß (SE) [a]	*p value*	ß (SE) [a]	*p value*	ß (SE) [a]	*p value*
Total problems	*Referent*	1.0 (3.7)	0.78	5.6 (6.4)	0.39	14.3 (6.1)	0.02
Internalizing	*Referent*	-0.2 (1.3)	0.89	0.9 (2.2)	0.69	5.6 (2.1)	0.01
Externalizing	*Referent*	0.3 (1.3)	0.83	1.3 (2.3)	0.59	4.1 (2.2)	0.06
Narrow-band syndromes							
Emotionally reactive	*Referent*	-0.4 (0.4)	0.38	0.5 (0.8)	0.49	1.8 (0.7)	0.02
Anxious/depressed	*Referent*	0.3 (0.4)	0.53	0.5 (0.7)	0.47	2.1 (0.7)	< 0.01
Somatic complaints	*Referent*	0.1 (0.4)	0.85	0.5 (0.7)	0.47	0.8 (0.6)	0.20
Withdrawn	*Referent*	-0.1 (0.4)	0.77	-0.6 (0.6)	0.30	0.8 (0.6)	0.17
Attention problems	*Referent*	0.2 (0.3)	0.52	0.7 (0.6)	0.25	0.6 (0.5)	0.30
Aggressive behavior	*Referent*	0.1 (1.1)	0.96	0.6 (1.9)	0.76	3.6 (1.8)	0.05
Sleep problems	*Referent*	0.5 (0.4)	0.24	0.6 (0.7)	0.39	0.5 (0.4)	0.59

Abbreviation: ß, Beta-coefficient; SE, standard error

[a] Beta-coefficient of combined effect of mercury levels and the APOE genotype (reference: non 4 carrier with lower Hg in cord blood); Model adjusted for participants' sex, birth order, cotinine, lead and selenium levels in cord blood, HOME, birth weight, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption.

Table 3S- 1 Subject characteristics and birth outcome of TBPS cohort and present study

	TBPS cohort			Present study		
	N (%)	CB Hg		N (%)	CB Hg	
		Mean (SD)	*p* value		Mean (SD)	*p* value
Sex						
Male	230 (51.1)	14.8 (9.5)	0.12	95 (57.2)	15.2 (8.7)	0.39
Female	220 (48.9)	13.4 (8.6)		71 (42.7)	14.0 (8.8)	
Birth Order						
1	220 (48.9)	13.7 (9.2)	0.41	72 (43.4)	13.8 (8.3)	0.27
>=2	230 (51.1)	14.4 (9.0)		94 (56.6)	15.3 (9.0)	
Birth Outcome						
Preterm						
Yes	38 (8.4)	13.1 (11.1)	0.49	13 (7.8)	12.9 (5.9)	0.45
No	412 (91.5)	14.2 (8.9)		153 (92.2)	14.8 (8.9)	
Low birth weight						
Yes	25 (5.0)	11.6 (6.8)	0.16	11 (6.6)	13.8 (7.0)	0.72
No	425 (95.0)	14.2 (9.2)		155 (93.4)	14.7 (8.9)	
Small for gestation age						
Yes	30 (6.7)	13.8 (9.4)	0.86	6 (3.6)	18.8 (11.1)	0.24
No	420 (93.3)	14.1 (9.1)		160 (96.4)	14.5 (8.6)	

Table 3S- 2 Maternal characteristic of subject in TBPS cohort and present study

	TBPS cohort			Present study		
	N (%)	CB Hg		N (%)	CB Hg	
		Mean (SD)	*p* value		Mean (SD)	*p* value
Age (%)						
<25	54 (12.0)	11.4 (7.2)	<0.01	10 (6.0)	11.8 (6.5)	0.03
25-35	309 (68.7)	13.3 (7.7)		121 (72.9)	13.9 (8.4)	
>=35	87 (19.3)	18.6 (12.7)		35 (21.1)	18.0 (9.8)	
Maternal Education						
< High school	45 (10.0)	9.6 (6.6)	<0.01	9 (5.4)	9.6 (5.4)	0.16
High School	211 (46.9)	13.8 (8.7)		68 (41.0)	14.4 (8.4)	
College and above	194 (43.1)	15.5 (9.7)		89 (53.6)	15.4 (9.1)	
Household income						
At birth			0.01			0.02
<1,000,000	272 (60.4)	13.2 (8.7)		85 (51.2)	13.1 (8.1)	
>=1,000,000	178 (39.6)	15.4 (9.5)		81 (48.8)	16.3 (9.1)	
At 2 years of age [a]			<0.01			<0.01
<1,000,000	109(45.8)	13.04 (7.7)		77 (46.4)	12.8 (7.3)	
>=1,000,000	129 (54.2)	16.94 (9.9)		89 (53.6)	16.3 (9.5)	

Table 3S- 3 Prenatal environmental exposure characteristic during pregnancy in TBPS cohort and present study

	TBPS cohort			Present study		
	N (%)	CB Hg		N (%)	CB Hg	
		Mean (SD)	*p* value		Mean (SD)	*p* value
Prenatal ETS			0.52			
No	320 (71.1)	14.3 (9.5)		123 (74.1)	14.3 (8.6)	0.34
Yes	130 (28.9)	13.7 (8.0)		43 (25.9)	15.8 (9.1)	
Fish Consumption [a]			0.56			0.30
No	21 (4.7)	13.0 (9.1)		5 (3.0)	10.7 (10.3)	
Yes	429 (95.3)	14.2 (9.1)		161 (97.0)	14.8 (8.7)	
Marine fish consumption			<0.01 [b]			0.03 [b]
0	39 (8.7)	12.3 (9.0)		10 (6.0)	11.5 (10.5)	
<1 ounce/week	108 (24.0)	12.6 (6.9)		43 (25.9)	14.3 (8.5)	
>=1 & <2 ounce/week	71 (15.8)	13.2 (7.1)		33 (19.9)	12.9 (7.6)	
>=2 and <7 ounce/week	169 (37.6)	15.0 (9.9)		55 (33.1)	14.5 (8.9)	
>=7 ounce/week	60 (13.3)	16.8 (11.5)		25 (15.1)	19.1 (8.6)	
Mising	3 (0.7)	7.8 (2.3)				

[a] Maternal fish consumption include marine and fresh water fish during pregnancy

[b] *p* for trends

Table 3S- 4 Mean and Standard Deviation (SD) of CBCL scores of participants at age 2 year old by APOE genotypes and cord blood Hg levels and stratified by sex

	Boy					Girl				
	All	Non ε4 carrier		ε4 carrier		All	Non ε4 carrier		ε4 carrier	
		<12ug/l	>12ug/l	<12ug/l	>12ug/l		<12ug/l	>12ug/l	<12ug/l	>12ug/l
Number of subjects	95	37	45	6	7	71	31	26	6	8
Child Behavior Checklist Mean (SD)										
Total Score	49.9 (21.8)	48.6 (22.7)	49.9 (19.9)	54.2 (31.1)	53.3 (8.4)	42.2 (21.8)	42.8 (19.4)	49.9 (19.9)	46.2 (20.1)	52.1 (22.4)
Internalizing	13.0 (7.8)	12.8 (7.3)	12.8 (7.7)	13.8 (10.5)	15.4 (3.0)	10.5 (6.5)	10.9 (6.1)	12.8 (7.7)	10.3 (6.9)	15.1 (8.0)
Externalizing	17.1 (7.6)	16.5 (7.8)	17.3 (7.2)	17.7 (9.0)	15.4 (3.0)	14.1 (7.6)	14.6 (8.0)	17.3 (7.2)	16.0 (7.8)	15.9 (7.5)
Narrow-band syndromes										
Emotionally reactive	3.4 (2.6)	3.3 (2.6)	3.2 (2.5)	4.2 (3.2)	4.3 (1.0)	2.7 (2.4)	2.9 (2.4)	3.2 (2.5)	2.8 (2.3)	4.4 (2.9)
Anxious/depressed	4.0 (2.4)	3.8 (2.2)	4.1 (2.5)	4.7 (2.9)	4.4 (0.9)	3.3 (2.1)	3.3 (2.1)	4.1 (2.5)	3.2 (1.7)	5.4 (2.3)
Somatic complaints	3.0 (2.3)	2.9 (2.2)	2.9 (2.3)	3.3 (3.3)	3.3 (0.9)	2.5 (1.9)	2.5 (1.9)	2.9 (2.3)	2.8 (2.2)	3.1 (2.3)
Withdrawn	2.6 (2.2)	2.8 (2.0)	2.6 (2.3)	1.7 (1.6)	3.4 (0.8)	2.0 (1.7)	2.1 (1.6)	2.6 (2.3)	1.5 (1.6)	2.3 (2.2)
Attention problems	3.2 (1.9)	3.2 (2.0)	3.1 (1.7)	3.7 (2.9)	3.7 (0.7)	2.6 (1.8)	2.5 (1.8)	3.1 (1.7)	3.2 (1.7)	2.0 (1.2)
Aggressive behavior	13.9 (6.3)	13.3 (6.3)	14.2 (6.0)	14.0 (6.4)	14.7 (2.4)	11.5 (6.5)	12.1 (7.0)	14.2 (6.0)	12.8 (6.7)	13.9 (6.7)
Sleep problems	4.3 (2.4)	4.3 (2.5)	4.5 (2.2)	4.5 (3.3)	3.1 (0.9)	4.5 (2.4)	4.3 (2.4)	4.5 (2.2)	4.8 (2.1)	5.0 (2.0)

Table 3S- 5 Multiple linear regression of CBCL scores of participants at age 2 year old by APOE genotypes and cord blood Hg levels and stratified by sex

	Boy				Girl			
	Non e4 carrier		e4 carrier		Non e4 carrier		e4 carrier	
	<12ug/l	>12ug/l	<12ug/l	>12ug/l	<12ug/l	>12ug/l	<12ug/l	>12ug/l
	37	45	6	7	31	26	6	8
Child Behavior Checklist		B (SE)	B (SE)	B (SE)		B (SE)	B (SE)	B (SE)
Child Behavior Checklist	*Referent*	4.7 (5.0)	7.8 (9.7)	14.0 (9.8)	*Referent*	-2.0 (5.8)	2.1 (9.1)	11.6 (8.4)
Internalizing	*Referent*	1.3 (1.8)	1.7 (3.5)	5.9 (3.5)	*Referent*	-1.7 (1.8)	-1.4 (2.9)	4.9 (2.7)
Externalizing	*Referent*	1.5 (1.8)	1.8 (3.4)	5.4 (3.4)	*Referent*	-0.7 (2.2)	1.2 (3.5)	1.8 (3.2)
Narrow-band syndromes								
Emotionally reactive	*Referent*	0.1 (0.6)	1.5 (1.2)	1.8 (1.2)	*Referent*	-1.0 (0.7)	-0.4 (1.1)	1.5 (1.0)
Anxious/depressed	*Referent*	0.7 (0.6)	0.7 (1.1)	1.6 (1.1)	*Referent*	-0.2 (0.6)	0.0 (0.9)	2.4 (0.9)*
Somatic complaints	*Referent*	0.4 (0.6)	0.5 (1.1)	0.8 (1.1)	*Referent*	-0.3 (0.6)	-0.4 (0.9)	0.8 (0.8)
Withdrawn	*Referent*	0.1 (0.5)	-0.9 (1.0)	1.7 (1.0)	*Referent*	-0.3 (0.5)	-0.6 (0.8)	0.2 (0.8)
Attention problems	*Referent*	0.2 (0.5)	0.5 (0.9)	1.3 (0.9)	*Referent*	0.3 (0.5)	0.7 (0.8)	-0.5 (0.7)
Aggressive behavior	*Referent*	1.3 (1.4)	1.3 (2.8)	4.1 (2.8)	*Referent*	-1.0 (1.9)	0.5 (2.9)	2.3 (2.7)
Sleep problems	*Referent*	0.5 (0.5)	0.9 (1.1)	-0.9 (1.1)	*Referent*	0.7 (0.7)	1.0 (1.2)	1.2 (1.1)

[a] Beta-coefficient of combined effect of mercury levels and APOE genotype (reference: non 4 carrier with lower Hg in cord blood); Model adjusted for participants' birth order, cotinine, lead and selenium level in cord blood, HOME, birth weight, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption.

**p<0.01*

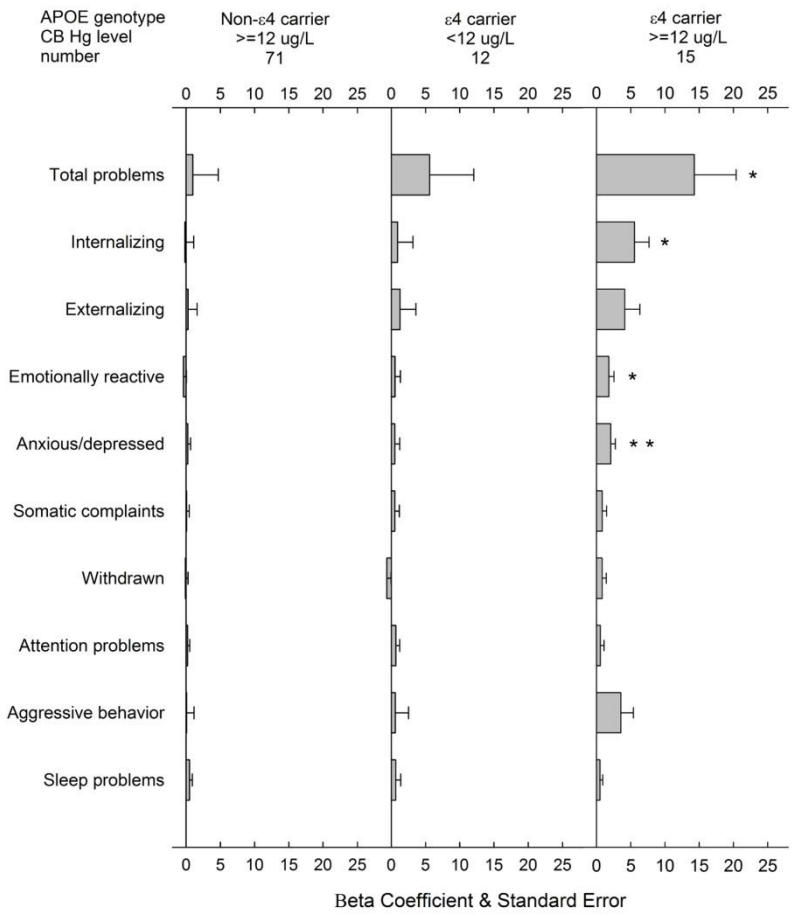
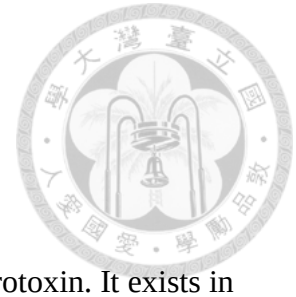


Figure 3-1 Multiple linear regression of CBCL by Cord blood mercury level (<12ug/L Vs >=12ug/L) and APOE genotype (ε4 carrier Vs non ε4 carrier). Model adjusted for participants' sex, birth order, cotinine, lead and selenium level in cord blood, birth weight, maternal age, maternal education level, maternal pregnancy marine fish consumption, HOME and family income.

*p<0.05 ; **p<0.01*



Chapter Four: Prenatal Mercury Exposure and Child Growth

4.1 Introduction

Mercury (Hg), a heavy metal, is a confirmed teratogen and neurotoxin. It exists in the environment in three major forms: organic, metallic and elemental. Because of its characteristics of high bioaccumulation and bio-magnification, methyl mercury (MeHg) is the primary and most toxic form of mercury that humans are exposed to. In addition to environmental exposure, MeHg is approximately 95 to 100% absorbed by the gastrointestinal tract. MeHg is able to pass through the placental blood barrier and foetal blood brain barrier, accumulate in brain tissue, and adversely affect foetal brain development or cause brain damage (Cernichiari et al., 1995, Clarkson and Magos, 2006, Farina et al., 2011, Kerper et al., 1992, Sakamoto et al., 2004, Simmons-Willis et al., 2002, Yee and Choi, 1996). However, results on the negative impacts of prenatal mercury exposure on child health are inconsistent in fish eating populations. A main reason for this inconsistency is the beneficial nutrients, such as poly unsaturated fatty acids, which partially compensate for Hg's harmful effect.

The impacts of prenatal mercury exposure on birth outcomes in fish eating populations remain unclear. Previous studies investigating the relationship between prenatal Hg exposure and birth outcomes have shown mixed results. A recent Spanish cohort study demonstrated that cord blood mercury was negatively associated with cord

blood Hg levels after adjusting for covariates, including fish consumption (Ramon et al., 2009). However, no association between birth weight and prenatal Hg exposure was demonstrated in cohort studies in France (Drouillet-Pinard et al., 2010), New York (Lederman et al., 2008), Canada (Lucas et al., 2004) and the United Kingdom (Daniels et al., 2007). A Denmark cohort study first demonstrated that cord blood mercury concentration was associated with body weight reduction in infants at 18 months old (Grandjean et al., 2003). A recent South Korean cohort study also reported that late pregnancy and cord blood Hg were associated with infant weight gain at 12 months and 24 months of age without considering fish consumption (Kim et al., 2011). However, the duration of this negative impact of prenatal Hg exposure on child growth remains unclear.

Hg has been recognized as a weak oestrogen mimic. A review article has concluded that Hg affects the endocrine system by the following possible mechanisms: accumulating in the endocrine system, having specific cytotoxicity in endocrine tissue, altering hormone concentration, interacting with sex hormones, and up/down-regulating enzyme activity of steroidogenesis (National Health Council, 2000, Tan et al., 2009). In rodents, females were found to have higher body concentrations of MeHg than males (National Health Council, 2000, Nielsen and Andersen, 1991a, b, Thomas et al., 1986, Thomas et al., 1987). The role of gender difference in prenatal susceptibility to prenatal

mercury exposure on child growth is unknown. Therefore, the aims of present study are to investigate the relationship between cord blood Hg concentration and child growth from birth to 9 years of ages and to examine the presence of gender difference in prenatal susceptibility to Hg exposure.

4.2 Materials and Methods

Study population

This study was an investigation of the Taiwan Birth Panel Study (TBPS). A total of 486 pregnant women and their neonates were recruited between April 2004 and January 2005 from one medical hospital in Taipei city, one area hospital, and two clinics in Taipei County. Informed consent was obtained by interview from mothers before delivery. Mothers were interviewed by trained interviewers using a structured questionnaire to collect the demographic characteristics, environmental exposure and family medication history before postpartum discharge. Then, maternal blood (10 ml) and cord blood (10 ml) were collected at delivery. The bloods were separated into whole blood, plasma and DNA. All of the samples were stored at -80℃ until being processed for laboratory analysis.

We continued following our study participants at different ages. We collected their health related information including medical history, life style related behaviour, and home and parenting related information by mail questionnaire and face to face interviews at 4, 6, 12, 24, 60, 84 and 108 months of age. The study subjects were restricted to those with cord blood Hg (N=450) and who responded to the follow up (N=321). Finally, 321 total subjects with 3970 measurements were included in this study. All protocols used in recruitment and follow-up periods were approved by the

Ethical Committee and Institutional Review Board of National Taiwan University Hospital.

Exposure assessment

Mercury level in cord blood was used to demonstrate the status of prenatal Hg exposure in participants. Whole blood was collected in ethylene-diamine-tetraacetic acid (EDTA) tubes and stored at -80℃ until the laboratory analysis was conducted. The analysis of 18 metals including Hg in blood was performed by the Agilent 7500C inductively coupled plasma mass spectrometer (ICP-MS) (Barany et al., 1997). For the experiment's quality assurance and quality control, 1 spike per 10 samples was applied to ensure the measurements were reliable. The detection limit of Hg in the present study was 0.18 μg/L, and the cord blood Hg levels of all participants were detectable.

Outcome measurement

The birth outcomes examined in this study included birth weight (grams), birth length (centimetres), and head circumference (centimetres). All of the data at birth were obtained from medical records. Then, body mass index (BMI, kg/m^2) was calculated. Additionally, gestational age was calculated by obstetricians based on last menstruation or early ultrasound estimates.

The child growth data examined in this study included body weight (kg), body length/height (centimetres) and head circumference (centimetres). All the growth data were obtained from the Child Healthcare Handbooks, which was published and designed by the Ministry of Health and Welfare of Taiwan to record information on

child-rearing, vaccination, and health check-up. Nine free health examinations were provided for each child from birth to 7 years old in Taiwan. The body weight and length/height of the child were measured and recorded in this handbook at each health examination or outpatient clinic visit.

Covariates

The risk factors that could interfere with the relationship between prenatal Hg exposure and child growth were considered. The following covariates were controlled for in the present study: infant sex, gestational age (<37 and ≥37 weeks), birth order (1st, 2nd and above), cotinine (log, ng/mL) in cord blood, maternal age (<25, 25 to 35 and ≥ 35 years old), maternal education level (≤Junior high school, high school, ≥college), maternal pregnancy marine fish consumption (no, ≤ 1 ounces/ week, ≥1 and <2 ounces/ week, 2 to <7 ounce/week, ≥7ounce/ week), breastfeeding (never/ever) and family income (<1,000,000 New Taiwan dollars, ≥1,000,000 New Taiwan dollars). This information was collected via the structured questionnaires.

Statistical analysis

Blood Hg concentrations were logarithm 10 transformed, and the participants were categorized into 4 groups according to their cord blood Hg levels by percentile of <25, 25-50, 50-75 and ≥75 to assess the dose-response relationship between blood Hg concentration and birth outcomes and child growth. The growth data were analysed at

the following time span: at birth, 0 to 6 months of age, 6 to 12 months of age, 12 to 24 months of age, 24 to 60 months of age and 60 to 108 months of age. Additionally, analysis was conducted from birth to the following age endpoints: 1 year old, 2 years old, 5 years old and 9 years old.

The age-specific z-scores for weight, length/height, BMI, weight for length and head circumference were calculated by WHO anthroplus software. The outliers were excluded in the analysis following the instructions of the WHO. To understand the relationship between the demographic characteristics and exposure, as well as birth outcomes, the mean value of blood Hg concentrations and birth outcomes with each demographic were estimated.

For birth outcomes, simple and multiple linear regressions were used to assess the association between cord blood mercury concentration and birth outcomes. The covariates selected in multiple regression analysis were infant sex, gestational age, birth order, maternal age at delivery, maternal education, family income, maternal marine fish consumption during pregnancy and blood cotinine concentration.

Because the time points and time intervals of the repeated measurements in this study were irregular, a mixed model with a flexible approach was applied to assess the effect of prenatal Hg exposure on child growth from birth to 9 years old. Univariate and multivariate analyses were performed to assess the relationship between cord blood Hg

concentration and each growth indicator. The covariates selected for multivariate analysis were infant sex, gestational age, birth order, maternal age at delivery, maternal education, breastfeeding, family income, maternal marine fish consumption during pregnancy and blood cotinine concentration.

Finally, gender specific analyses were performed. We stratified subjects by gender to estimate the gender specific effect of blood Hg concentration on growth. Univariate and multivariate regressions were applied to estimate the association between blood Hg concentration and birth outcomes by sex. Univariate and multivariate mixed models were used to calculate the association between blood Hg concentration and child growth. All analyses were conducted using SPSS 16.0 and SAS Version 9.3. (SAS Institute Inc. Cary, NC, USA).

4.3 Results

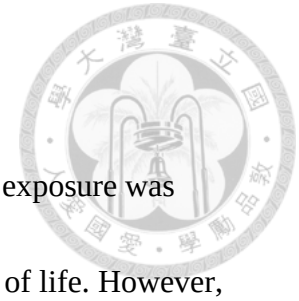
Table 4-1 demonstrates the mean value of cord blood Hg, maternal blood Hg, cord blood cotinine concentration and birth outcomes of by participants, non-participants and both sexes. Higher cord blood Hg concentrations were found in participants, and the higher cord blood cotinine was found in non-participants. Birth outcomes of participants were comparable or even better than those of non-participants. For the participant, boys had higher cord blood Hg and cotinine concentrations but better birth outcomes compared to girls.

The demographic characteristics of participants and non- participants in both sexes are shown in table 4-2. Mothers from non-participants were younger participants. Higher proportions of primipara mothers (54.3%), preterm birth (13.2%) and small for gestational age (9.5%) were found in non-participants. Better social economic status (maternal education and annual family income) was found in participants. The characteristics were similar between both sexes.

The association between cord blood hg concentration and birth outcomes are shown in table 4-3. No consistent associations between Hg concentration and birth outcomes were found in the univariate analysis. After adjusting for the confounding factors, we found that z-score of the birth weight and birth length were inversely associated with

cord blood Hg concentrations. Additionally, consistent findings were found in the dose dependent test, and significance was indicated by $p < 0.05$.

Table 4-4 shows the relationship between cord blood Hg concentration and child growth. Cord blood Hg concentrations were negatively associated with the z-score of all growth indicators before and after adjusting for confounding factors. The z-score of weight, weight for length and BMI significantly decreased with the increase of cord blood Hg concentrations in children 12 -24 months old. The consistent findings were also found in the dose dependent test (p for trend <0.05, shown in table 4S1-1, 2, 3. For the gender specific analysis, a negative association between cord blood Hg and child growth was found, and statistically significant association was found in girls. We found that the z-scores of body weight, length/ height, BMI, weight for length/height significantly decreased with the increase in cord blood Hg concentration in girls, and this impact was found until 2 years of age. Furthermore, cord blood Hg was also found to be borderline significant in girls' body weight at ages 2-5 years old and 5-9 years old. Additionally, all of the above associations were significant in the dose dependent test, shown in table 4S3-1, 2, 3.



4.4 Discussion

In the present study, we found that the adverse effect of prenatal Hg exposure was associated with child body weight and body mass in the first 2 years of life. However, after stratified analysis, we found that the impact was only significant among female children. We found that prenatal Hg concentration was associated with decreased head circumference from birth to 6 months of age, and body weight, body length and body mass in girls aged 0 to 2 years old. Moreover, although the negative effect became slightly significant at ages 2 to 9 years old, and the results of the dose dependent relationship test suggested that the effect remained. Therefore, we suggested that prenatal Hg exposure may negatively affect child growth, and girls are more susceptible to this effect.

The biological mechanism of the mercury effects at birth and during infant growth remains unclear, although there are several possible pathways. A possible explanation is that Hg interferes with intracellular calcium homeostasis (Sirois and Atchison, 2000) and impairs foetal growth. Additionally, Hg can interact with glutathione and decrease the levels of glutathione (Shanker et al., 2005). The depletion of glutathione may increase the levels of reactive oxygen species oxidative stress and contribute to mitochondrial dysfunction (Ou et al., 1999). It has been suggested that MeHg can alter the activities of the Glutathione-related enzymes, glutathione peroxidase and glutathione

reductase (Farina et al., 2005, Farina et al., 2003, Stringari et al., 2006). Moreover, an in vivo study has suggested that Hg can inhibit the mammalian thioredoxin system. Thioredoxin reductase and thioredoxin are widely distributed in various mammalian organs and tissues. Thioredoxin reductase, similar to glutathione peroxidase, is responsible for catalysing redox reactions involved in fundamental mechanisms in cell growth, such as DNA synthesis and antioxidant defence, and is critical for cellular stress response, protein repair, and protection against oxidative damage(Carvalho et al., 2008, Papp et al., 2007, Rozell et al., 1985).

Sex differences in susceptibility to Hg have been observed and discussed in both human and animal studies. An observational study that followed the sex ratio of the Minamata disaster reported a decrease in the sex ratio and an increase in stillbirth in males and male adult mortality(Sakamoto et al., 2001). A decrease in the male-to-female sex ratio was also observed in fish and rodent studies (Matta et al., 2001, Vorhees, 1985). Although the impact of Hg on sex ratio was consistent across species, it is certainly attributed to a high concentration of Hg exposure and is difficult to extrapolate to the general population. Sex-specific differences in Hg accumulation, Hg elimination and Hg renal toxicity have been found in rodents. For instance, male mice accumulate higher levels of Hg in the kidney and are more sensitive to renal toxicity of Hg. A possible reason for this phenomenon is that oestrogen can increase glutathione synthesis by the

liver and induce the secretion of Hg from the kidney (National Health Council, 2000, Tan et al., 2009). Two studies demonstrated that the patterns of Hg accumulation and excretion and renal effect of castrated male mice were similar to females. Moreover, other studies used synthesised testosterone and estradiol to demonstrate the role of sex hormone on Hg toxicity and suggested that this difference results from sex-related differences in glutathione metabolism in the kidney and liver (Hirano et al., 1988, Hirayama et al., 1987). Male mice tended to accumulate more Hg in the kidney, and females tended to accumulate more Hg in the brain. Therefore, male rodents were more susceptible to Hg exposure in the kidney, which may contribute to the accumulation of more Hg in the kidney and vice versa. Numerous potential mechanisms, such as the effect on sex hormones, may be directly or indirectly associated with sex specific differences in the susceptibility to Hg in foetuses and children. However, the mechanisms associated with sex differences in susceptibility to prenatal Hg exposure on both human and animal offspring growth are unknown. More studies are needed to investigate the sex-specific differences in susceptibility to Hg.

A limitation of this study was the moderate response rate, and the demographic variables between respondents and non-respondents may lead to a selection bias. The differences include higher blood Hg, older maternal age range but better birth outcomes, higher education level and higher household income in participants. However, a higher

socioeconomic level is correlated with providing better resources for the child. Therefore, our results may not be overestimated. Second, although polychlorinated biphenyl (PCB), a hormone disrupting agent that humans may be exposed to via fish consumption, was not investigated. The blood PCB concentration in Taiwanese pregnant women is relatively low (Wang et al., 2004), and there were no reports of high PCB exposure via fish in Taiwan. Therefore, the claim that the lack of exposure information of PCB may interfere with our findings was limited.

Although some limitations were present, the present study was able to adequately provide scientific finding regarding the effect of Hg on child growth and the gender specific differences in the susceptibility to prenatal Hg exposure for the following reasons. First, we used cord blood Hg, which is a reliable biomarker for prenatal Hg exposure. Second, the birth outcomes and multiple child growth measurements were reliable. Additionally, the information on the potential confounders was accurate and comprehensive, as the exposure levels of cotinine were directly measured in cord blood. These advantages minimized the possibility of recall bias and misclassification.

In conclusion, we found that girls may be more susceptible to prenatal Hg exposure than boys, and this effect was found to be significant in the first two years of life. This effect lasts until nine years of age. Fish consumption is a primary source of Hg exposure worldwide. However, fish consumption should not be avoided due to potential mercury

exposure, because fish provide abundant essential nutrients and proteins for living needs. Guidelines and interventions to reduce mercury exposure through fish eating are necessary, especially for children and pregnant women. Additionally, more toxicology studies are needed to understand the mechanism of sex-specific susceptibility to mercury in children.

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Table 4- 1 Characteristics of environmental exposure and birth outcomes of study population

	Non-participants	Participants		
		All	Boy	Girl
Subject No.	N=129	N=321	N=173	N=148
Exposure characteristics				
Cord Blood Hg (ng/mL)	12.1 (8.6)	14.9 (9.2)	15.7 (9.9)	13.9 (8.2)
Maternal Blood Hg (ng/mL)	5.7 (4.8)	7.3 (5.1)	7.6 (5.1)	6.9 (5.0)
Cord Blood Cotinine	5.9 (23.9)	2.1 (16.1)	2.7 (20.8)	1.5 (9.1)
Birth outcomes				
Gestational age (weeks)	38.1 (1.5)	38.7 (1.6)	38.7 (1.6)	38.7 (1.7)
Birth weight (grams)	3054.4 (424.9)	3206.7 (464.1)	3276.3 (456.2)	3125.4 (461.6)
Birth length (cm)	48.8 (2.2)	49.1 (2.1)	49.4 (2.0)	48.8 (2.2)
Head circumference (cm)	32.8 (1.7)	33.9 (1.5)	34.1 (1.4)	33.5 (1.5)
Body Mass Index (kg/m^2)	12.7 (1.1)	13.2 (1.3)	13.4 (1.3)	13.1 (1.3)

Table 4- 2 Demographic characteristic of participants, non-participants and both sexes

	Non-participants	Participants		
Characteristics		All	Boy	Girl
Subject No.	N=129	N=321	N=173	N=148
Birth Order				
1	70 (54.3)	150 (46.7)	80 (46.2)	70 (47.3)
>=2	59 (45.7)	171 (53.3)	93 (53.8)	78 (52.7)
Preterm (%)				
Yes	17 (13.2)	21 (6.5)	11 (6.4)	10 (6.8)
No	112 (86.8)	300 (93.5)	162 (93.6)	138 (93.2)
Low birth weight				
Yes	6 (4.7)	19 (5.9)	8 (4.6)	11 (7.4)
No	122 (95.3)	302 (94.1)	165 (95.4)	137 (92.6)
Birth size (%)				
SGA	12 (9.5)	18 (5.6)	7 (4.1)	11 (7.4)
AGA	107 (84.3)	268 (83.5)	144 (83.2)	124 (83.8)
LGA	8 (6.3)	35 (10.9)	22 (12.7)	13 (8.8)
Breast-Feeding				
NO	-	31 (13.0)	18 (10.4)	13 (8.8)
Ever	-	208 (87.0)	114 (65.9)	94 (63.5)
missing	-	82 (25.6)	41 (23.7)	41 (27.7)
Maternal Age (%)				
<25	34 (26.4)	20 (6.2)	11 (6.4)	9 (6.1)
25-35	86 (66.7)	223 (69.5)	118 (68.2)	105 (71.0)
>=35	9 (7.0)	78 (24.3)	44 (25.4)	34 (23.0)
Maternal Education				
Junior high school	29 (22.5)	16 (5.0)	5 (2.9)	11 (7.4)
High School	81 (62.8)	130 (40.5)	76 (43.9)	54 (36.5)
College and above	19 (14.7)	175 (54.5)	92 (53.2)	83 (56.1)
Marine Fish (ounces/week)				
No	12 (9.3)	32 (10.0)	15 (8.7)	17 (11.5)
<1	32 (24.8)	76 (23.7)	43 (24.9)	33 (22.3)
>=1 & <2	20 (15.5)	50 (15.6)	29 (16.7)	21 (14.2)
>=2 and <7	47 (36.4)	121 (37.7)	61 (35.3)	60 (40.5)
>=7	18 (14.0)	42 (13.1)	25 (14.4)	17 (11.5)
Annual family income				
< NT 1,000,000	96 (74.4)	176 (54.8)	102 (59.0)	74 (50.0)
>=NT 1,000,000	33 (25.6)	145 (45.2)	71 (41.0)	74 (50.0)

Abbreviation: SGA, Small for Gestational Age; AGA, Appropriate for Gestational Age; LGA, Large for gestational age; NT: New Taiwan dollars

Table 4- 3 Regression coefficient and standard error of multivariable linear model of birth outcomes by cord blood mercury concentration and grouped mercury levels

	Birth weight		Birth length		Body Mass Index		Head circumference	
	Crude	Adjusted	Crude	Adjusted	Crude	Adjusted	Crude	Adjusted
				ß±SE				
All subject [a]	-0.01 (0.22)	-0.20 (0.19)	0.005 (0.22)	-0.09 (0.21)	0.02 (0.22)	-0.19 (0.21)	0.07 (0.24)	-0.20 (0.23)
Boys [b]	0.28 (0.29)	0.13 (0.26)	0.49 (0.30)	0.43 (0.28)	0.03 (0.31)	-0.15 (0.30)	0.28 (0.33)	<0.001 (0.32)
Girls [b]	-0.38 (0.33)	**-0.56 (0.27)***	-0.50 (0.32)	**-0.64 (0.30)***	-0.05 (0.31)	-0.27 (0.28)	-0.16 (0.36)	-0.35 (0.35)
Dose dependent tests								
All subjects [a]								
> 8.63-12.25	-0.04 (-0.22)	-0.13 (0.14)	-0.05 (0.16)	-0.11 (0.15)	-0.02 (0.16)	-0.11 (0.15)	0.04 (0.18)	-0.08 (0.17)
>12.25-19.54	-0.26 (-1.61)	-0.18 (0.14)	-0.30 (0.16)	-0.18 (0.15)	-0.07 (0.16)	-0.05 (0.15)	-0.19 (0.18)	-0.22 (0.17)
>19.54	0.00 (-0.02)	-0.15 (0.14)	-0.03 (0.16)	-0.12 (0.15)	0.02 (0.16)	-0.12 (0.15)	0.06 (0.18)	-0.12 (0.17)
p for trend	0.646	0.273	0.507	0.373	0.990	0.530	0.951	0.374
Boys [b]								
> 8.63-12.25	0.05 (0.22)	0.01 (0.19)	0.02 (0.23)	-0.02 (0.21)	0.05 (0.24)	0.01 (0.23)	-0.02 (0.25)	-0.19 (0.24)
>12.25-19.54	-0.04 (0.22)	0.06 (0.20)	-0.05 (0.23)	0.09 (0.21)	-0.04 (0.24)	0.01 (0.23)	0.05 (0.25)	-0.01 (0.24)
>19.54	0.19 (0.22)	0.09 (0.20)	0.33 (0.23)	0.26 (0.22)	0.02 (0.24)	-0.08 (0.23)	0.12 (0.25)	-0.10 (0.25)
p for trend	0.478	0.581	0.186	0.153	0.947	0.715	0.551	0.934
Girls [b]								
> 8.63-12.25	-0.10 (0.24)	-0.18 (0.20)	-0.03 (0.23)	-0.08 (0.21)	-0.12 (0.23)	-0.21 (0.20)	0.13 (0.26)	0.10 (0.25)
>12.25-19.54	**-0.49 (0.24)***	-0.39 (0.20)	**-0.49 (0.23)***	**-0.44 (0.22)***	-0.14 (0.23)	-0.13 (0.21)	-0.44 (0.26)	-0.47 (0.25)
>19.54	-0.21 (0.24)	-0.37 (0.20)	-0.40 (0.23)	**-0.53 (0.22)***	-0.003 (0.23)	-0.13 (0.21)	0.02 (0.26)	-0.14 (0.25)
p for trend	0.162	**0.035**	**0.03**	**0.007**	0.909	0.560	0.541	0.272

[a] Model adjusted for infant sex, birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

[b] Model adjusted for birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

Reference group, cord blood Hg ≤ 8.63 ng/mL; * *p<0.05*

Table 4- 4 Regression coefficient and standard error of multivariable mixed model of z-score of growth indicator by cord blood mercury concentration

		Follow-up stage								
		0-6 m	6-12 m	12-24 m	24-60 m	60-108 m	0-12 m	0-24 m	0-60 m	0-108 m
Weight										
All	Crude	-0.24 (0.20)	-0.29 (0.23)	**-0.58 (0.23)***	-0.22 (0.26)	-0.26 (0.33)	-0.28 (0.19)	**-0.42 (0.19)***	-0.34 (0.19)	-0.31 (0.19)
	Adjusted[a]	-0.35 (0.20)	-0.36 (0.24)	**-0.68 (0.25)***	-0.37 (0.27)	-0.26 (0.36)	-0.38 (0.20)	**-0.51 (0.20)***	**-0.43 (0.20)***	**-0.40 (0.20)***
Boys	Crude	0.01 (0.29)	-0.19 (0.33)	-0.39 (0.35)	-0.11 (0.40)	0.07 (0.51)	-0.08 (0.29)	-0.24 (0.29)	-0.23 (0.29)	-0.15 (0.29)
	Adjusted[b]	-0.14 (0.30)	-0.24 (0.37)	-0.39 (0.39)	-0.30 (0.45)	0.07 (0.57)	-0.18 (0.31)	-0.29 (0.31)	-0.30 (0.31)	-0.28 (0.31)
Girls	Crude	-0.51 (0.26)	-0.41 (0.30)	**-0.80 (0.29)***	-0.34 (0.31)	-0.73 (0.43)	-0.49 (0.25)	**-0.61 (0.24)***	-0.46 (0.25)	**-0.50 (0.25)***
	Adjusted[b]	**-0.63 (0.24)***	-0.55 (0.31)	**-1.08 (0.29)***	-0.57 (0.32)	-0.81 (0.45)	**-0.64 (0.24)***	**-0.80 (0.24)***	**-0.62 (0.24)***	**-0.61 (0.25)***
Height										
All	Crude	-0.02 (0.24)	-0.06 (0.24)	-0.43 (0.24)	-0.09 (0.24)	-0.23 (0.27)	-0.06 (0.22)	-0.23 (0.21)	-0.19 (0.20)	-0.19 (0.20)
	Adjusted[a]	-0.21 (0.24)	-0.05 (0.25)	-0.48 (0.25)	-0.23 (0.25)	-0.26 (0.29)	-0.19 (0.22)	-0.34 (0.21)	-0.29 (0.21)	-0.29 (0.20)
Boys	Crude	0.26 (0.37)	0.22 (0.34)	-0.26 (0.35)	0.01 (0.36)	0.18 (0.37)	0.21 (0.33)	-0.01 (0.31)	-0.04 (0.30)	0.01 (0.29)
	Adjusted[b]	-0.15 (0.38)	0.05 (0.38)	-0.36 (0.39)	-0.27 (0.40)	-0.04 (0.41)	-0.08 (0.34)	-0.23 (0.33)	-0.24 (0.32)	-0.21 (0.31)
Girls	Crude	-0.28 (0.31)	-0.27 (0.34)	-0.46 (0.31)	-0.08 (0.31)	-0.55 (0.41)	-0.31 (0.29)	-0.40 (0.27)	-0.28 (0.27)	-0.33 (0.27)
	Adjusted[b]	-0.49 (0.29)	-0.28 (0.35)	-0.63 (0.32)	-0.37 (0.31)	-0.63 (0.41)	-0.46 (0.28)	**-0.56 (0.26)***	-0.43 (0.26)	-0.48 (0.26)

[a] Model adjusted for infant sex, birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

[b] Model adjusted for birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption;

** $p<0.05$*

Table 4-4 Regression coefficient and standard error of multivariable mixed model of z-score of growth indicator by cord blood mercury concentration *(continuous)*

		Follow-up stage								
		0-6 m	6-12 m	12-24 m	24-60 m	60-108 m	0-12 m	0-24 m	0-60 m	0-108 m
BMI										
All	Crude	-0.30 (0.20)	-0.42 (0.23)	-0.41 (0.23)	-0.21 (0.27)	-0.22 (0.35)	-0.34 (0.19)	**-0.37 (0.18)***	-0.31 (0.18)	-0.26 (0.18)
	Adjusted[a]	-0.33 (0.21)	**-0.52 (0.25)***	-0.47 (0.24)	-0.32 (0.29)	-0.10 (0.37)	-0.38 (0.20)	**-0.41 (0.20)***	-0.35 (0.20)	-0.31 (0.20)
Boys	Crude	-0.16 (0.29)	-0.51 (0.33)	-0.31 (0.34)	-0.12 (0.41)	-0.14 (0.55)	-0.27 (0.28)	-0.30 (0.27)	-0.26 (0.28)	-0.21 (0.28)
	Adjusted[b]	-0.09 (0.32)	-0.49 (0.37)	-0.22 (0.38)	-0.21 (0.46)	0.07 (0.61)	-0.21 (0.30)	-0.23 (0.30)	-0.25 (0.31)	-0.25 (0.31)
Girls	Crude	-0.48 (0.27)	-0.39 (0.32)	**-0.64 (0.29)***	-0.38 (0.33)	-0.55 (0.43)	-0.45 (0.25)	**-0.51 (0.24)***	-0.43 (0.24)	-0.41 (0.23)
	Adjusted[b]	-0.56 (0.28)	-0.59 (0.34)	**-0.83 (0.31)***	-0.48 (0.35)	-0.59 (0.46)	**-0.59 (0.27)***	**-0.64 (0.25)***	**-0.52 (0.25)***	-0.45 (0.24)
Weight for length										
All	Crude	-0.32 (0.21)	-0.43 (0.23)	**-0.47 (0.23)***	-0.21 (0.27)		-0.36 (0.19)	**-0.40 (0.18)***	-0.33 (0.18)	
	Adjusted[a]	-0.30 (0.23)	**-0.52 (0.24)***	**-0.53 (0.25)***	-0.32 (0.29)		-0.38 (0.20)	**-0.42 (0.19)***	-0.37 (0.20)	
Boys	Crude	-0.29 (0.31)	-0.50 (0.32)	-0.34 (0.35)	-0.13 (0.43)		-0.35 (0.28)	-0.36 (0.27)	-0.30 (0.28)	
	Adjusted[b]	-0.11 (0.34)	-0.48 (0.36)	-0.26 (0.39)	-0.20 (0.48)		-0.23 (0.31)	-0.27 (0.30)	-0.26 (0.31)	
Girls	Crude	-0.39 (0.29)	-0.41 (0.32)	**-0.71 (0.29)***	-0.38 (0.32)		-0.42 (0.25)	**-0.51 (0.24)***	-0.43 (0.23)	
	Adjusted[b]	-0.42 (0.31)	-0.61 (0.33)	**-0.91 (0.30)***	-0.49 (0.34)		-0.53 (0.27)	**-0.62 (0.25)***	**-0.52 (0.25)***	

[a] Model adjusted for infant sex, birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

[b] Model adjusted for birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

** $p<0.05$*

Table 4-4 Regression coefficient and standard error of multivariable mixed model of z-score of growth indicator by cord blood mercury concentration *(continuous)*

		Follow-up stage								
		0-6 m	6-12 m	12-24 m	24-60 m	60-108 m	0-12 m	0-24 m	0-60 m	0-108 m
Head circumference										
All	Crude	-0.28 (0.22)	0.03 (0.22)	-0.11 (0.21)			-0.19 (0.20)	-0.25 (0.20)		
	Adjusted[a]	-0.38 (0.23)	-0.09 (0.24)	-0.18 (0.23)			-0.28 (0.21)	-0.32 (0.21)		
Boys	Crude	-0.09 (0.31)	0.07 (0.32)	0.17 (0.30)			-0.04 (0.29)	-0.04 (0.28)		
	Adjusted[b]	-0.10 (0.33)	-0.08 (0.35)	0.03 (0.32)			-0.06 (0.31)	-0.09 (0.30)		
Girls	Crude	-0.49 (0.31)	-0.05 (0.31)	-0.34 (0.30)			-0.39 (0.28)	-0.48 (0.27)		
	Adjusted[b]	**-0.62 (0.30)***	-0.07 (0.32)	-0.40 (0.32)			-0.46 (0.29)	-0.55 (0.28)		

[a] Model adjusted for infant sex, birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

[b] Model adjusted for birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

** $p<0.05$*

Table 4S1- 1 Regression coefficient and standard error of multivariable mixed model of z-score of growth indicator by grouped cord blood mercury levels: body weight and body height (All subjects)

	Follow-up stage								
All subjects	0-6 m	6-12 m	12-24 m	24-60 m	60-108 m	0-12 m	0-24 m	0-60 m	0-108 m
Weight									
Crude									
2nd quartile	-0.21 (0.15)	-0.18 (0.17)	**-0.39 (0.17)***	-0.25 (0.20)	-0.12 (0.26)	-0.23 (0.14)	**-0.29 (0.14)***	**-0.29 (0.14)***	-0.22 (0.14)
3rd quartile	**-0.37 (0.15)***	**-0.37 (0.17)***	**-0.41 (0.18)***	-0.19 (0.19)	0.03 (0.25)	**-0.37 (0.15)***	**-0.41 (0.14)***	**-0.38 (0.15)***	**-0.29 (0.14)***
4th quartile	-0.12 (0.15)	-0.14 (0.17)	**-0.37 (0.17)***	-0.19 (0.19)	-0.24 (0.25)	-0.15 (0.14)	-0.24 (0.14)	-0.20 (0.14)	-0.18 (0.14)
p for trend	0.287	0.268	**0.038**	0.385	0.458	0.196	0.065	0.123	0.178
Adjusted[a]									
2nd quartile	-0.24 (0.14)	-0.22 (0.17)	**-0.43 (0.17)***	-0.31 (0.20)	-0.18 (0.27)	**-0.28 (0.14)***	**-0.33 (0.14)***	**-0.32 (0.14)***	-0.26 (0.14)
3rd quartile	**-0.32 (0.15)***	-0.33 (0.18)	**-0.39 (0.18)***	-0.21 (0.20)	0.03 (0.27)	**-0.33 (0.15)***	**-0.37 (0.15)***	**-0.34 (0.15)***	-0.26 (0.15)
4th quartile	-0.20 (0.14)	-0.20 (0.18)	**-0.43 (0.18)***	-0.27 (0.20)	-0.25 (0.27)	-0.23 (0.14)	**-0.30 (0.14)***	-0.26 (0.15)	-0.24 (0.15)
p for trend	0.175	0.258	**0.034**	0.264	0.501	0.131	**0.049**	0.106	0.144
Height									
Crude									
2nd quartile	-0.19 (0.18)	**-0.38 (0.18)***	**-0.42 (0.18)***	-0.23 (0.18)	-0.20 (0.22)	-0.32 (0.16)	**-0.40 (0.15)***	**-0.37 (0.15)***	**-0.32 (0.15)***
3rd quartile	-0.33 (0.19)	**-0.42 (0.19)***	-0.30 (0.18)	-0.18 (0.18)	-0.08 (0.21)	**-0.37 (0.17)***	**-0.37 (0.16)***	**-0.34 (0.16)***	-0.28 (0.15)
4th quartile	0.01 (0.18)	-0.09 (0.18)	-0.33 (0.18)	-0.09 (0.18)	-0.24 (0.21)	-0.07 (0.16)	-0.19 (0.16)	-0.16 (0.15)	-0.15 (0.15)
p for trend	0.863	0.626	0.119	0.666	0.352	0.638	0.269	0.367	0.384
Adjusted[a]									
2nd quartile	-0.28 (0.17)	**-0.38 (0.18)***	**-0.44 (0.18)***	-0.28 (0.19)	-0.22 (0.22)	**-0.38 (0.16)***	**-0.45 (0.15)***	**-0.42 (0.15)***	**-0.37 (0.14)***
3rd quartile	-0.31 (0.18)	-0.31 (0.19)	-0.25 (0.19)	-0.23 (0.19)	-0.03 (0.22)	-0.32 (0.17)	**-0.32 (0.16)***	-0.29 (0.16)	-0.23 (0.15)
4th quartile	-0.15 (0.18)	-0.10 (0.19)	**-0.37 (0.18)***	-0.17 (0.19)	-0.25 (0.22)	-0.17 (0.16)	-0.27 (0.16)	-0.22 (0.15)	-0.21 (0.15)
p for trend	0.441	0.809	0.121	0.445	0.419	0.436	0.197	0.297	0.312

[a] Model adjusted for sex birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

Cord blood mercury (ng/mL) of each group: Reference group, <8.63 ; 2nd quartile, > 8.63-12.25 ;3rd quartile, >12.25-19.54; 4th quartile, >19.54

** p<0.05*

Table 4S1- 2 Regression coefficient and standard error of multivariable mixed model of z-score of growth indicator by grouped cord blood mercury levels: body mass index and weight for length (All subjects)

	Follow-up stage								
All subjects	0-6 m	6-12 m	12-24 m	24-60 m	60-108 m	0-12 m	0-24 m	0-60 m	0-108 m
BMI									
Crude									
2nd quartile	-0.13 (0.15)	0.05 (0.17)	-0.24 (0.17)	-0.25 (0.21)	-0.0031 (0.28)	-0.06 (0.14)	-0.10 (0.14)	-0.13 (0.14)	-0.08 (0.14)
3rd quartile	-0.24 (0.15)	-0.12 (0.18)	-0.33 (0.17)	-0.10 (0.20)	0.11 (0.27)	-0.18 (0.14)	-0.22 (0.14)	-0.21 (0.14)	-0.14 (0.14)
4th quartile	-0.16 (0.15)	-0.17 (0.17)	-0.23 (0.17)	-0.20 (0.20)	-0.17 (0.27)	-0.17 (0.14)	-0.19 (0.14)	-0.18 (0.14)	-0.14 (0.14)
p for trend	0.229	0.219	0.151	0.446	0.581	0.157	0.114	0.161	0.272
Adjusted[a]									
2nd quartile	-0.13 (0.15)	-0.01 (0.18)	-0.28 (0.17)	-0.32 (0.21)	-0.07 (0.28)	-0.08 (0.14)	-0.11 (0.14)	-0.15 (0.14)	-0.10 (0.14)
3rd quartile	-0.21 (0.16)	-0.13 (0.19)	-0.34 (0.18)	-0.12 (0.21)	0.07 (0.28)	-0.17 (0.15)	-0.21 (0.15)	-0.20 (0.15)	-0.14 (0.15)
4th quartile	-0.17 (0.16)	-0.23 (0.18)	-0.26 (0.18)	-0.27 (0.21)	-0.18 (0.28)	-0.20 (0.15)	-0.21 (0.15)	-0.20 (0.15)	-0.16 (0.14)
p for trend	0.251	0.150	0.163	0.348	0.606	0.152	0.124	0.161	0.257
Weight for Length									
Crude									
2nd quartile	-0.08 (0.16)	0.03 (0.17)	-0.30 (0.17)	-0.20 (0.21)	-	-0.04 (0.14)	-0.10 (0.14)	-0.13 (0.14)	-
3rd quartile	-0.13 (0.16)	-0.14 (0.18)	**-0.38 (0.17)***	-0.11 (0.20)	-	-0.13 (0.15)	-0.22 (0.14)	-0.21 (0.14)	-
4th quartile	-0.17 (0.16)	-0.18 (0.17)	-0.27 (0.17)	-0.19 (0.20)	-	-0.18 (0.14)	-0.21 (0.14)	-0.20 (0.14)	-
p for trend	0.271	0.190	0.105	0.433	-	0.156	0.082	0.128	-
Adjusted[a]									
2nd quartile	-0.06 (0.16)	-0.02 (0.17)	**-0.35 (0.17)***	-0.27 (0.21)		-0.05 (0.14)	-0.11 (0.14)	-0.14 (0.14)	
3rd quartile	-0.12 (0.17)	-0.15 (0.19)	**-0.38 (0.18)***	-0.12 (0.22)		-0.13 (0.15)	-0.20 (0.15)	-0.20 (0.15)	
4th quartile	-0.14 (0.17)	-0.24 (0.18)	-0.31 (0.18)	-0.27 (0.21)		-0.18 (0.15)	-0.22 (0.14)	-0.21 (0.15)	
p for trend	0.384	0.141	0.114	0.332		0.188	0.107	0.141	

[a] Model adjusted for sex birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

Cord blood mercury (ng/mL) of each group: Reference group, <8.63 ; 2nd quartile, > 8.63-12.25 ;3rd quartile, >12.25-19.54; 4th quartile, >19.54

** p<0.05*

Table 4S1- 3 Regression coefficient and standard error of multivariable mixed model of z-score of growth indicator by grouped cord blood mercury levels: head circumference (All subjects)

	Follow-up stage								
All subjects	0-6 m	6-12 m	12-24 m	24-60 m	60-108 m	0-12 m	0-24 m	0-60 m	0-108 m
Head circumference									
Crude									
2nd quartile	-0.28 (0.17)	-0.11 (0.16)	-0.30 (0.15)	-	-	-0.20 (0.15)	**-0.30 (0.14)***	-	-
3rd quartile	**-0.38 (0.17)***	-0.14 (0.17)	**-0.32 (0.16)***	-	-	-0.30 (0.16)	**-0.39 (0.15)***	-	-
4th quartile	-0.17 (0.17)	0.08 (0.17)	-0.04 (0.15)	-	-	-0.09 (0.15)	-0.12 (0.15)	-	-
p for trend	0.255	0.695	0.743	-	-	0.473	0.329		
Adjusted[a]									
2nd quartile	**-0.32 (0.16)***	-0.14 (0.17)	**-0.34 (0.16)***	-	-	-0.24 (0.15)	-0.33 (0.15)	-	-
3rd quartile	-0.33 (0.17)	-0.13 (0.18)	**-0.35 (0.17)***	-	-	-0.27 (0.16)	-0.37 (0.16)	-	-
4th quartile	-0.25 (0.17)	-0.004 (0.18)	-0.09 (0.16)	-	-	-0.15 (0.16)	-0.18 (0.15)	-	-
p for trend	0.170	0.973	0.626	-	-	0.369	0.279	-	-

[a] Model adjusted for sex birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

Cord blood mercury (ng/mL) of each group: Reference group, <8.63 ; 2nd quartile, > 8.63-12.25 ;3rd quartile, >12.25-19.54; 4th quartile, >19.54

** p<0.05*

Table 4S2- 1 Regression coefficient and standard error of multivariable mixed model of z-score of growth indicator by grouped cord blood mercury levels: body weight and body height (Boys)

	Follow-up stage								
Boys	0-6 m	6-12 m	12-24 m	24-60 m	60-108 m	0-12 m	0-24 m	0-60 m	0-108 m
Weight									
Crude									
2nd quartile	-0.21 (0.23)	-0.17 (0.26)	-0.35 (0.27)	-0.39 (0.31)	-0.11 (0.43)	-0.22 (0.22)	-0.27 (0.22)	-0.28 (0.22)	-0.21 (0.22)
3rd quartile	-0.25 (0.23)	-0.30 (0.27)	-0.28 (0.27)	0.00 (0.31)	0.39 (0.42)	-0.26 (0.22)	-0.29 (0.22)	-0.27 (0.22)	-0.15 (0.22)
4th quartile	0.01 (0.22)	-0.05 (0.26)	-0.18 (0.27)	-0.07 (0.30)	0.09 (0.41)	-0.04 (0.22)	-0.12 (0.22)	-0.12 (0.22)	-0.06 (0.22)
p for trend	0.910	0.870	0.680	0.797	0.543	0.939	0.712	0.728	0.930
Adjusted[a]									
2nd quartile	-0.23 (0.22)	-0.22 (0.27)	-0.34 (0.28)	-0.47 (0.32)	-0.08 (0.46)	-0.27 (0.22)	-0.30 (0.23)	-0.32 (0.23)	-0.27 (0.23)
3rd quartile	-0.28 (0.23)	-0.25 (0.29)	-0.22 (0.30)	-0.03 (0.33)	0.46 (0.47)	-0.26 (0.23)	-0.25 (0.24)	-0.24 (0.24)	-0.17 (0.24)
4th quartile	-0.08 (0.23)	-0.11 (0.29)	-0.18 (0.30)	-0.21 (0.33)	0.15 (0.46)	-0.12 (0.23)	-0.16 (0.23)	-0.18 (0.24)	-0.16 (0.24)
p for trend	0.841	0.851	0.799	0.968	0.520	0.808	0.701	0.665	0.721
Height									
Crude									
2nd quartile	-0.48 (0.28)	**-0.51 (0.26)** *	**-0.52 (0.26)***	**-0.55 (0.27)***	-0.31 (0.31)	**-0.51 (0.24)***	**-0.56 (0.23)***	**-0.55 (0.23)***	**-0.46 (0.22)***
3rd quartile	-0.38 (0.28)	-0.39 (0.27)	-0.34 (0.26)	-0.21 (0.27)	0.08 (0.30)	-0.36 (0.25)	-0.37 (0.24)	-0.33 (0.23)	-0.22 (0.22)
4th quartile	0.17 (0.28)	0.08 (0.26)	-0.12 (0.26)	0.00 (0.26)	0.09 (0.30)	0.10 (0.24)	-0.04 (0.23)	-0.04 (0.23)	-0.01 (0.22)
p for trend	0.364	0.401	0.989	0.536	0.311	0.383	0.667	0.687	0.571
Adjusted[a]									
2nd quartile	**-0.65 (0.27)***	**-0.61 (0.27)***	**-0.60 (0.27)***	**-0.67 (0.28)***	-0.38 (0.33)	**-0.66 (0.24)***	**-0.68 (0.24)***	**-0.67 (0.23)***	**-0.59 (0.22)***
3rd quartile	-0.53 (0.28)	-0.43 (0.29)	-0.33 (0.29)	-0.34 (0.29)	-0.04 (0.34)	-0.45 (0.25)	-0.42 (0.25)	-0.37 (0.24)	-0.30 (0.23)
4th quartile	-0.15 (0.28)	-0.07 (0.28)	-0.27 (0.28)	-0.27 (0.29)	-0.10 (0.33)	-0.14 (0.25)	-0.23 (0.25)	-0.24 (0.24)	-0.20 (0.23)
p for trend	0.980	0.591	0.802	0.847	0.746	0.812	0.941	0.918	0.978

[a] Model adjusted for birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

Cord blood mercury (ng/mL) of each group: Reference group, <8.63 ; 2nd quartile, > 8.63-12.25 ;3rd quartile, >12.25-19.54; 4th quartile, >19.54

** p<0.05*

Table 4S2- 2 Regression coefficient and standard error of multivariable mixed model of z-score of growth indicator by grouped cord blood mercury levels: body mass index and weight for length (Boys)

	Follow-up stage								
	0-6 m	6-12 m	12-24 m	24-60 m	60-108 m	0-12 m	0-24 m	0-60 m	0-108 m
BMI									
Crude									
2nd quartile	0.07 (0.22)	0.15 (0.25)	-0.13 (0.26)	-0.19 (0.32)	0.08 (0.46)	0.08 (0.21)	0.04 (0.21)	0.00 (0.21)	0.03 (0.21)
3rd quartile	-0.06 (0.23)	-0.02 (0.26)	-0.12 (0.26)	0.21 (0.32)	0.48 (0.45)	-0.04 (0.22)	-0.07 (0.21)	-0.06 (0.22)	0.02 (0.22)
4th quartile	-0.09 (0.22)	-0.17 (0.25)	-0.15 (0.25)	-0.10 (0.31)	0.01 (0.44)	-0.14 (0.21)	-0.14 (0.21)	-0.14 (0.21)	-0.10 (0.22)
p for trend	0.540	0.316	0.594	0.932	0.908	0.385	0.395	0.460	0.610
Adjusted[a]									
2nd quartile	0.17 (0.23)	0.18 (0.27)	-0.05 (0.28)	-0.22 (0.33)	0.21 (0.49)	0.14 (0.22)	0.11 (0.22)	0.05 (0.22)	0.06 (0.23)
3rd quartile	0.03 (0.24)	0.04 (0.28)	-0.04 (0.29)	0.21 (0.34)	0.69 (0.51)	0.04 (0.23)	0.03 (0.23)	0.01 (0.23)	0.05 (0.23)
4th quartile	0.00 (0.24)	-0.12 (0.28)	-0.04 (0.29)	-0.13 (0.34)	0.25 (0.49)	-0.07 (0.23)	-0.05 (0.23)	-0.10 (0.23)	-0.08 (0.24)
p for trend	0.760	0.424	0.929	0.953	0.564	0.564	0.641	0.590	0.658
Weight for Length									
Crude									
2nd quartile	0.22 (0.23)	0.12 (0.25)	-0.22 (0.26)	-0.14 (0.33)		0.16 (0.21)	0.06 (0.21)	0.04 (0.21)	
3rd quartile	0.07 (0.24)	-0.04 (0.26)	-0.18 (0.27)	0.17 (0.33)		0.03 (0.22)	-0.05 (0.21)	-0.04 (0.22)	
4th quartile	-0.16 (0.24)	-0.16 (0.25)	-0.17 (0.26)	-0.09 (0.32)		-0.17 (0.21)	-0.17 (0.21)	-0.15 (0.21)	
p for trend	0.325	0.343	0.621	0.991		0.277	0.304	0.391	
Adjusted[a]									
2nd quartile	0.38 (0.24)	0.15 (0.26)	-0.15 (0.28)	-0.17 (0.34)		0.25 (0.22)	0.15 (0.22)	0.09 (0.22)	
3rd quartile	0.18 (0.26)	0.02 (0.28)	-0.10 (0.29)	0.22 (0.36)		0.13 (0.23)	0.06 (0.23)	0.04 (0.23)	
4th quartile	0.03 (0.26)	-0.12 (0.28)	-0.07 (0.29)	-0.12 (0.36)		-0.05 (0.23)	-0.06 (0.23)	-0.10 (0.23)	
p for trend	0.703	0.458	0.911	0.966		0.538	0.579	0.564	

[a] Model adjusted for birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

Cord blood mercury (ng/mL) of each group: Reference group, <8.63 ; 2nd quartile, > 8.63-12.25 ;3rd quartile, >12.25-19.54; 4th quartile, >19.54

** $p<0.05$*

Table 4S2- 3 Regression coefficient and standard error of multivariable mixed model of z-score of growth indicator by grouped cord blood mercury levels: head circumference (Boys)

	Follow-up stage								
	0-6 m	6-12 m	12-24 m	24-60 m	60-108 m	0-12 m	0-24 m	0-60 m	0-108 m
Head circumference									
Crude									
2nd quartile	-0.47 (0.24)	-0.14 (0.24)	-0.20 (0.22)			-0.33 (0.22)	-0.35 (0.21)		
3rd quartile	-0.33 (0.24)	-0.11 (0.25)	0.05 (0.23)			-0.28 (0.23)	-0.24 (0.22)		
4th quartile	-0.09 (0.24)	0.13 (0.25)	0.16 (0.22)			0.01 (0.23)	0.04 (0.21)		
p for trend	0.992	0.499	0.232			0.771	0.580		
Adjusted[a]									
2nd quartile	-0.47 (0.24)	-0.20 (0.25)	-0.31 (0.23)			-0.36 (0.23)	-0.40 (0.22)		
3rd quartile	-0.24 (0.25)	-0.04 (0.26)	-0.03 (0.24)			-0.17 (0.24)	-0.19 (0.23)		
4th quartile	-0.13 (0.25)	-0.002 (0.27)	-0.004 (0.24)			-0.06 (0.24)	-0.05 (0.23)		
p for trend	0.951	0.720	0.522			0.768	0.688		

[a] Model adjusted for birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

Cord blood mercury (ng/mL) of each group: Reference group, <8.63 ; 2nd quartile, > 8.63-12.25 ;3rd quartile, >12.25-19.54; 4th quartile, >19.54

** p<0.05*

Table 4S3- 1 Regression coefficient and standard error of multivariable mixed model of z-score of growth indicator by grouped cord blood mercury levels: body weight and body height (Girls)

	Follow-up stage								
Girls	0-6 m	6-12 m	12-24 m	24-60 m	60-108 m	0-12 m	0-24 m	0-60 m	0-108 m
Weight									
Crude									
2nd quartile	-0.13 (0.20)	-0.18 (0.23)	-0.41 (0.21)	-0.08 (0.24)	-0.04 (0.31)	-0.19 (0.19)	-0.27 (0.18)	-0.28 (0.18)	-0.22 (0.18)
3rd quartile	**-0.46 (0.20)***	-0.41 (0.23)	**-0.49 (0.22)***	-0.33 (0.23)	-0.35 (0.31)	**-0.45 (0.19)***	**-0.50 (0.18)***	**-0.47 (0.18)***	**-0.42 (0.18)***
4th quartile	-0.25 (0.19)	-0.26 (0.23)	**-0.59 (0.22)***	-0.29 (0.24)	**-0.66 (0.31)***	-0.27 (0.19)	**-0.37 (0.18)***	-0.28 (0.18)	-0.31 (0.18)
p for trend	0.078	0.144	**0.005**	0.122	**0.025**	0.058	**0.016**	0.059	**0.047**
Adjusted[a]									
2nd quartile	-0.18 (0.18)	-0.24 (0.23)	**-0.47 (0.21)***	-0.22 (0.25)	-0.21 (0.33)	-0.25 (0.17)	-0.32 (0.17)	-0.32 (0.18)	-0.27 (0.18)
3rd quartile	**-0.43 (0.18)***	-0.40 (0.24)	**-0.62 (0.22)***	-0.41 (0.23)	-0.40 (0.31)	**-0.43 (0.18)***	**-0.50 (0.18)***	**-0.45 (0.18)***	**-0.37 (0.18)***
4th quartile	-0.33 (0.18)	-0.34 (0.23)	**-0.73 (0.21)***	-0.42 (0.23)	**-0.67 (0.32)***	**-0.38 (0.18)***	**-0.48 (0.17)***	**-0.37 (0.18)***	**-0.36 (0.18)***
p for trend	**0.030**	0.100	**<0.001**	**0.049**	**0.031**	**0.018**	**0.003**	**0.021**	**0.032**
Height									
Crude									
2nd quartile	0.21 (0.23)	-0.14 (0.26)	-0.23 (0.23)	0.29 (0.25)	0.06 (0.30)	-0.03 (0.22)	-0.14 (0.20)	-0.11 (0.20)	-0.09 (0.20)
3rd quartile	-0.23 (0.23)	-0.34 (0.26)	-0.11 (0.23)	-0.08 (0.23)	-0.17 (0.30)	-0.33 (0.22)	-0.29 (0.20)	-0.27 (0.20)	-0.26 (0.20)
4th quartile	-0.20 (0.23)	-0.27 (0.26)	**-0.50 (0.23)***	-0.05 (0.23)	**-0.60 (0.30)***	-0.27 (0.22)	-0.35 (0.20)	-0.24 (0.20)	-0.29 (0.20)
p for trend	0.189	0.200	0.061	0.590	**0.043**	0.112	0.054	0.157	0.092
Adjusted[a]									
2nd quartile	0.10 (0.21)	-0.24 (0.26)	-0.28 (0.22)	0.10 (0.25)	-0.17 (0.30)	-0.13 (0.20)	-0.22 (0.19)	-0.19 (0.19)	-0.18 (0.19)
3rd quartile	-0.26 (0.22)	-0.17 (0.27)	-0.11 (0.24)	-0.18 (0.23)	-0.20 (0.29)	-0.28 (0.22)	-0.25 (0.20)	-0.23 (0.20)	-0.21 (0.19)
4th quartile	-0.35 (0.21)	-0.30 (0.26)	**-0.59 (0.23)***	-0.26 (0.23)	**-0.60 (0.29)***	-0.39 (0.21)	**-0.47 (0.19)***	-0.36 (0.19)	**-0.39 (0.19)***
p for trend	0.055	0.264	**0.028**	0.1885	**0.048**	**0.049**	**0.018**	0.063	**0.044**

[a] Model adjusted for birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

Cord blood mercury (ng/mL) of each group: Reference group, <8.63 ; 2nd quartile, > 8.63-12.25 ;3rd quartile, >12.25-19.54; 4th quartile, >19.54

** p<0.05*

Table 4S3- 2 Regression coefficient and standard error of multivariable mixed model of z-score of growth indicator by grouped cord blood mercury levels: body mass index and weight for length (Girls)

	Follow-up stage								
Girls	0-6 m	6-12 m	12-24 m	24-60 m	60-108 m	0-12 m	0-24 m	0-60 m	0-108 m
BMI									
Crude									
2nd quartile	-0.37 (0.20)	-0.13 (0.24)	-0.38 (0.21)	-0.40 (0.26)	-0.08 (0.31)	-0.25 (0.19)	-0.27 (0.18)	-0.32 (0.18)	-0.25 (0.17)
3rd quartile	**-0.45 (0.20)***	-0.24 (0.24)	**-0.60 (0.21)***	-0.39 (0.24)	-0.39 (0.31)	-0.35 (0.19)	**-0.41 (0.18)***	**-0.40 (0.18)***	**-0.35 (0.17)***
4th quartile	-0.20 (0.20)	-0.18 (0.24)	-0.36 (0.21)	-0.36 (0.25)	-0.42 (0.31)	-0.20 (0.19)	-0.25 (0.18)	-0.24 (0.18)	-0.21 (0.17)
p for trend	0.223	0.368	**0.037**	0.116	0.112	0.193	0.08	0.110	0.141
Adjusted[a]									
2nd quartile	-0.35 (0.20)	-0.16 (0.25)	**-0.44 (0.21)***	-0.44 (0.27)	-0.14 (0.33)	-0.25 (0.19)	-0.28 (0.18)	-0.31 (0.18)	-0.26 (0.17)
3rd quartile	**-0.50 (0.22)***	-0.35 (0.26)	**-0.74 (0.23)***	-0.42 (0.25)	-0.42 (0.32)	**-0.41 (0.20)***	**-0.48 (0.19)***	**-0.43 (0.19)***	-0.33 (0.18)
4th quartile	-0.23 (0.20)	-0.28 (0.25)	**-0.44 (0.22)***	-0.38 (0.25)	-0.45 (0.32)	-0.27 (0.19)	-0.31 (0.18)	-0.27 (0.18)	-0.21 (0.18)
p for trend	0.182	0.204	**0.019**	0.120	0.120	0.117	0.050	0.091	0.179
Weight for Length									
Crude									
2nd quartile	**-0.47 (0.21)***	-0.12 (0.24)	**-0.42 (0.21)***	-0.35 (0.25)		-0.31 (0.19)	-0.31 (0.17)	-0.34 (0.17)	
3rd quartile	-0.38 (0.21)	-0.25 (0.24)	**-0.62 (0.21)***	-0.37 (0.23)		-0.34 (0.19)	**-0.42 (0.18)***	**-0.40 (0.17)***	
4th quartile	-0.14 (0.21)	-0.21 (0.24)	**-0.43 (0.21)***	-0.35 (0.24)		-0.18 (0.19)	-0.26 (0.18)	-0.25 (0.17)	
p for trend	0.499	0.298	**0.017**	0.107		0.277	0.079	0.100	
Adjusted[a]									
2nd quartile	-0.41 (0.22)	-0.16 (0.25)	**-0.49 (0.21)***	-0.41 (0.26)		-0.30 (0.19)	-0.31 (0.18)	-0.33 (0.18)	
3rd quartile	-0.43 (0.23)	-0.35 (0.26)	**-0.75 (0.22)***	-0.41 (0.24)		-0.41 (0.21)	**-0.48 (0.19)***	**-0.44 (0.18)***	
4th quartile	-0.12 (0.22)	-0.30 (0.25)	**-0.53 (0.22)***	-0.39 (0.25)		-0.21 (0.20)	-0.30 (0.18)	-0.27 (0.18)	
p for trend	0.505	0.165	**0.008**	0.098		0.215	0.060	0.088	

[a] Model adjusted for birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

Cord blood mercury (ng/mL) of each group: Reference group, <8.63 ; 2nd quartile, > 8.63-12.25 ;3rd quartile, >12.25-19.54; 4th quartile, >19.54

** p<0.05*

Table 4S3- 3 Regression coefficient and standard error of multivariable mixed model of z-score of growth indicator by grouped cord blood mercury levels: head circumference (Girls)

	Follow-up stage								
Girls	0-6 m	6-12 m	12-24 m	24-60 m	60-108 m	0-12 m	0-24 m	0-60 m	0-108 m
Head circumference									
Crude									
2nd quartile	-0.01 (0.23)	-0.08 (0.23)	-0.31 (0.21)			-0.03 (0.21)	-0.19 (0.20)		
3rd quartile	-0.44 (0.23)	-0.20 (0.23)	**-0.67 (0.22)***			-0.35 (0.21)	**-0.54 (0.20)***		
4th quartile	-0.29 (0.23)	-0.02 (0.24)	-0.17 (0.21)			-0.23 (0.21)	-0.30 (0.20)		
p for trend	0.082	0.754	0.144			0.1361	**0.045**		
Adjusted[a]									
2nd quartile	-0.01 (0.22)	-0.08 (0.23)	-0.29 (0.22)			-0.03 (0.21)	-0.18 (0.20)		
3rd quartile	**-0.48 (0.23)***	-0.13 (0.25)	**-0.72 (0.24)***			-0.35 (0.22)	**-0.54 (0.21)***		
4th quartile	-0.38 (0.22)	-0.08 (0.24)	-0.21 (0.23)			-0.29 (0.21)	-0.36 (0.20)		
p for trend	**0.033**	0.691	0.147			0.090	**0.032**		

[a] Model adjusted for birth order, log transformed cotinine concentration in cord blood, maternal age, maternal education level, family income and maternal pregnancy marine fish consumption

Cord blood mercury (ng/mL) of each group: Reference group, <8.63 ; 2nd quartile, > 8.63-12.25 ;3rd quartile, >12.25-19.54; 4th quartile, >19.54

** p<0.05*

Appendix

1. Mercury, APOE, and children's neurodevelopment.- published in Neurotoxicology 2013 Jul; 37: 85-92.

2. Mercury, APOE, and child behavior. --published in Chemosphere 2015 Feb; 120: 123-130.