Title
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Publication Date
2018

Peer reviewed|Thesis/dissertation

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By

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A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Epidemiology

in the

Graduate Division

of the

University of California, Berkeley

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Spring 2018

Abstract

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Evidence of inter-individual differences in toxicant response has necessitated heavy editing of Paracelsus' famous toxicological maxim "the dose makes the poison" to include factors such as age, sex, and timing of exposure. This dissertation takes advantage of a unique multigenerational, long-term cohort and advances in molecular technology to examine whether life stage and genetic factors also modify human sensitivity to toxic exposures, particularly with respect to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a persistent organic pollutant with well-documented carcinogenic and endocrine-disrupting effects in humans. Although increasing animal evidence supports the hypothesis that *in utero* exposure to endocrine disrupting compounds can have a long-term impact on the health of the 2nd and subsequent generations, the evidence in humans is limited. In addition, individuals may have differences in susceptibility to chemical exposure based on their genetic make-up.

On July 10, 1976, an explosion at a chemical plant near Seveso, Italy resulted in a toxic plume that exposed nearby residents to high levels of TCDD. The Seveso Women's Health Study (SWHS), an ambidirectional cohort study, was initiated in 1996 to investigate the health of 981 women who were newborn to age 40 years in 1976, had resided in the immediate vicinity of the plant, and had archived samples of blood collected soon after the explosion. The SWHS is the only comprehensive study of the health effects of TCDD exposure in a female population, and has the unique benefit of measurements of individual-level TCDD in blood collected near the time of the explosion. In 2014, 611 offspring of the SWHS who were born after the accident, and potentially exposed to their mother's TCDD body burdens *in utero* were enrolled.

The first two chapters examine the neurotoxic effects of TCDD during windows of susceptibility *in utero* and later in life when hormonal processes potentially sensitive to TCDD's estrogenic influence are driving brain changes. In particular, chapter 1 investigates TCDD and cognitive and physical functioning in SWHS women decades after their direct exposure to the accident and the modifying effects of menarche and menopause. Chapter 2 addresses the relation between neuropsychological function in 7-17 year old offspring (n=161) with respect to their mother's 1976 exposures and maternal levels estimated at the time of pregnancy. Lastly, Chapter 3 examines genetic susceptibility in the aryl hydrocarbon receptor (AhR), a key transcription factor in the metabolism of TCDD and other xenobiotics in humans. Specifically, we conducted a gene-by-environment (GxE) analysis to evaluate the modifying effect of genetic polymorphisms in maternal AhR on the relationship between maternal TCDD levels and child

birthweight, an indicator of a restricted fetal environment. The SWHS Second Generation Study in conjunction with the parent SWHS offers a rich dataset in which to explore windows of neurotoxic and genetic susceptibility to TCDD across the lifecourse and test the fetal origins of disease hypothesis

Table of Contents

I. DEDICATIONS

To my caring and funny parents, JoAnn and Chris. Thank you for passing along your love for learning and for your understanding when Boston got too snowy for me.

II. ACKNOWLEDGEMENTS

This dissertation, much like the best practices of causal inference, is more about the process that got me here ("the roadmap") than this final document. I have so many people to thank for giving me directions along the way. First, I want to thank my brilliant, savvy, and patient adviser and epidemiological hero, Professor Brenda Eskenazi, for showing me how to do a cohort study from soup to nuts (or, in Italian, antipasto a dolce) – it has been an invaluable experience to see the Seveso Second Generation study from its first days of funding to its first publications. I am also so grateful for her encouragement to think outside the box and the many opportunities to go to conferences, meet new people, and eat chocolate in her office. I also want to express my gratitude to Professor Nina Holland who took a risk on me when I wanted to switch gears and pursue molecular studies in the middle of my PhD. She welcomed me into her lab and through her devoted mentorship and crucial research insights, she taught me how to truly think like a scientist. I also want to thank Dr. Marcy Warner who played such an instrumental role in my development as an epidemiologist. She was my Seveso guru, providing extremely helpful feedback on all my analyses, presentations, and writing and we shared many good laughs throughout the process. I am also so appreciative of my committee members, professors Maya Petersen and Tyrone Hayes, who, in addition to providing helpful feedback, were exemplar educators – their engaging and clear teaching styles galvanized my interest in causal inference and endocrinology, respectively.

I was very fortunate to have had a supportive, hard-working, and inspiring community of classmates at UC Berkeley. It has been such a privilege to share these years with this talented group of friends and to discuss the intricacies of epidemiology and life over beers and home-baked goods. Thank you for the study sessions, happy hours, phenomenal feedback on papers and presentations, mock qualifying exams, and weekend writing retreats in Point Reyes (let's do it again!). I especially want to thank my PhD buddy Amelia Wallace who was so helpful in revising my F31 proposal and with whom I shared many commiseration sessions over momos at Tibet Café. I also want to thank Professor Jen Ahern for leading our doctoral seminar with so much dedication and for being like an auxiliary committee member to us all -- she reviewed all of our work with sincere interest and rigor and was key to promoting our dissertation progress and career development.

I am deeply appreciative for the support of the amazing research team at the Center for Environmental Research and Children's Health (CERCH). It was great to be in the company of so many smart, kind, and passionate people who see every research challenge as an opportunity. I also want to thank my colleagues at the Holland Lab at the Richmond Field Station for their guidance on molecular studies and for their contributions and good cheer during those tedious months of lab work, extracting DNA.

My friends and family were so unfailingly supportive during the ups and downs of the PhD experience. My parents and brothers were particularly important sources of humor and rejuvenation. With one brother just beginning a PhD and the other, highschool, I can only hope I left some parental patience untapped! I have so much gratitude especially to my mom who has masterfully balanced career and family and inspired me to enter public health and to my dad for tolerating our mother-daughter shoptalk and for always offering his judicious and calming advice when I needed it. I especially want to thank my friends Nami and Andrew for many nourishing home-cooked meals and weekend writing sojourns at their place in SF, my Frisbee teammates for giving me a reason to leave the office, my roommates for always being there with an open ear

and bottle of wine, my cousins at UC Berkeley who were always ready for family time, and my partner Jonathan who was so patient with me and kept me smiling (and also well-nourished!).

Most of all, I am indebted to the participants of the Seveso Women's Health Study who, despite tragic circumstances, have so generously shared their experiences so that we may learn as much as we can from the Seveso accident. We must not forget its legacy, especially as our world's relationship with chemicals grows more complex and fraught. It has been an honor to work on this study with our Italian colleagues Drs. Paolo Mocarelli, Paolo Brambilla, and Stefano Signorini, from whom I have learned so much.

I would also like to acknowledge the co-authors on my dissertation publications. Their expertise, study resources, feedback, and mentorship were integral to this work.

1. Neurocognitive and Physical Functioning in the Seveso Women's Health Study.
Ames J, Warner M, Brambilla P, Mocarelli P, Satariano WA, Eskenazi B.

Published in *Environmental Research.* Dec. 26, 2017

2. Prenatal Dioxin Exposure and Neuropsychological Functioning in the Seveso Second Generation Health Study.
Ames J, Warner M, Mocarelli P, Brambilla P, Signorini S, Siracusa C, Eskenazi B.

Submitted.

3. AHR Gene-Dioxin Interactions and Birthweight in the Seveso Second Generation Health Study.
Ames J, Warner M, Mocarelli P, Brambilla P, Signorini S, Huen K, Siracusa C, Holland N*, Eskenazi B*. (*joint senior authors)

Submitted.

III. AIMS AND ORGANIZATION OF DISSERTATION

This thesis investigates whether life-stage, sex, and genetics modify human sensitivity to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, a.k.a. dioxin), a widespread environmental contaminant of historical notoriety. We examine these factors in the Seveso Women's Health Study, the largest and most comprehensive study to-date of the health of women and children highly exposed to TCDD resulting from an industrial accident in 1976. Since 1996, the SWHS has followed the health of women in Seveso and has investigated the association between their 1976 exposures and many health outcomes of concern, including cancer, fertility, menstrual cycle characteristics, thyroid function, bone health, metabolic diseases, cardiovascular health, and pregnancy outcomes. Thus, the SWHS and their children represent a unique population with which to improve our understanding of the factors that influence sensitivity to toxic exposures and the longevity of these effects across generations. The three aims of this dissertation, specifically examining these factors in relation to neurodevelopment and fetal growth, are described below:

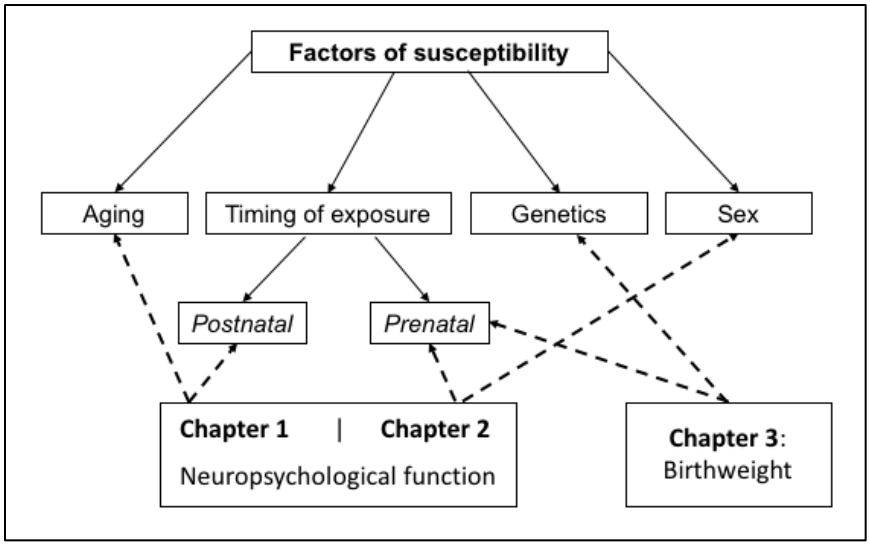

Figure 1. Schematic of dissertation aims (by chapter)

Specific Aims:

Aim 1 (Chapter 1): To determine the association between postnatal TCDD exposure on physical functioning and working memory in the SWHS women. Hypothesis: Higher serum concentrations of TCDD are inversely associated with performance on assessments of physical and cognitive functioning. We will examine the relationship between TCDD levels in 1976 and physical functioning in 154 women who completed an assessment in 1996 and working memory in 459 women who completed a neurocognitive assessment in 2008. In addition, we investigate whether this relationship is modified by periods of development and hormonal flux in the female life-course including menarche status at exposure and menopause status at outcome assessment.

Aim 2 (Chapter 2): **To determine the association between <u>prenatal TCDD</u> exposure and neuropsychological functioning in children of SWHS women**. This aim, in contrast to aim 1, investigates whether the brains of those exposed *in utero* may be more sensitive to TCDD exposure. <u>Hypothesis</u>: Higher *in utero* exposures will be associated with poorer performance on tasks of attention, memory, and learning ability in offspring. We will examine this association in an adolescent subset of second generation children (n=161) in the 2014 follow-up. Further, given the sexual patterning of brain development and the endocrine-disrupting properties of TCDD, this chapter also investigates whether these associations are modified by child sex and breastfeeding histories.

Aim 3 (Chapter 3): To examine <u>gene-environment interactions</u> between genetic variants in the aryl hydrocarbon receptor (*AHR*) and maternal levels of TCDD on birthweight. This chapter explores how single nucleotide polymorphisms (SNPs) in the receptor that binds TCDD and orchestrates its toxicity, modifies the association between the mother's 1976 exposure and fetal growth. <u>Hypothesis:</u> Genetic variants in the maternal *AHR* gene increase risk of lower birthweight in interaction with prenatal TCDD exposure. Independent and joint interactions of SNPs, combined in cumulative risk-allele scores, are evaluated.

IV. BACKGROUND AND SIGNIFICANCE

IV.I Overview – Dioxin

TCDD is a persistent organic pollutant with well-documented carcinogenic and endocrine-disrupting effects in humans and animals[1]. Historically formed as a chemically-stable by-product in combustive processes such as industrial manufacturing and, to a lesser degree, forest fires, TCDD remains ubiquitous in the environment, though environmental regulations have dramatically curbed its industrial release in recent decades. In 2001, the United Nation's Stockholm Convention, an international treaty on environmental protection, named TCDD as one of the "dirty dozen" – a priority list of ubiquitous persistent organic pollutants (POPs) threatening ecological and human health worldwide[2]. TCDD is also one of few chemicals to have the unequivocal classification as a known human carcinogen by the International Agency for Research on Cancer and the US National Toxicology Program [3,4]. TCDD, and related dioxin-like chemicals, bioaccumulate in the adipose tissue of animals and nearly 90% of human exposure to dioxins occurs through diet, particularly consumption of meat, dairy, and seafood. Given its chemical stability and lipophilicity, TCDD also has an exceptionally long half-life of 7-9 years in the human body.[5] While the detectable levels of dioxin found in most humans do not pose a health risk, aging populations with high fish and meat consumption are particularly vulnerable to accruing higher, potentially toxic body burdens over the life course. Further, low background levels of TCDD may still pose a risk to the developing fetus and infant through maternal-placental and lactational transfer.

IV.II Dioxin Toxicity via the Aryl Hydrocarbon Receptor

TCDD is the most toxic member of a class of planar halogenated hydrocarbons comprising polychlorinated dibenzo-para-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and the coplanar and mono-ortho-substituted congeners of polychlorinated biphenyls (dioxin-like PCBs). Known collectively as dioxins, these compounds share several chemical and, consequently, toxicological properties including their induction of the aryl hydrocarbon receptor (AhR). AhR is a nuclear receptor and transcription factor that regulates diverse physiological processes related to development, cell growth, apoptosis, immune function, and xenobiotic metabolism[6]. TCDD, as the most potent inducer of AhR, elicits the strongest AhR-mediated biochemical and toxic response in biological systems. It thus serves as the index chemical for determining the relative toxicities of other dioxins and dioxin-like compounds that are often summed together and reported as a total dioxin toxic equivalency (TEQ).

Upon binding TCDD in the cell's cytoplasm, AhR dimerizes with a co-factor protein, the Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT), and travels to the nucleus, where it induces expression of several xenobiotic metabolizing enzymes, such as cytochrome P4501A1 (CYP1A1), P4501A2 (CYP1A2), and P4501B1 (CYP1B1) and exhibits crosstalk with pathways of hormone synthesis. Despite over three decades of research on AhR and its relationship with TCDD, the exact mechanisms by which TCDD's unusually prolonged activation of AhR promotes toxicity remain poorly understood.[7] However, AhR is a highly conserved protein across the animal kingdom and a great deal of our understanding continues to emerge from studies of model organisms, including fish[8,9]. Such work, buttressed by a large body of experimental work *in vitro*, has shed light on AhR's myriad functions as a selectively-modulated receptor with activity and regulation differing by such factors as tissue type, ligand identity, and cellular availability of co-factor proteins; this complexity underscores the challenges of

understanding this fundamentally important transcription factor, particularly with respect to its mediation of toxic responses in humans [10,11].

IV.III Neurotoxic Effects of Dioxin

Animal studies have documented the neurotoxic effects of TCDD, dioxins, and related PCBs but few studies have investigated the exclusive effects of TCDD on the human brain.[12,13] One study of US veterans exposed to TCDD through the spraying of Agent Orange during the Vietnam War found that the highest exposed individuals performed more poorly on tests of verbal memory than unexposed peers.[14] A slightly larger literature exists on the neurotoxicity of PCB mixtures which, given the chemical similarities with TCDD, are suggestive of the otherwise under-documented neurotoxic potential of TCDD. For example, several studies have found associations between body PCB burdens and impairments in memory, learning, and executive functioning in adults.[15,16]

These neurological deficits have been more pronounced in groups such as children and older adults, perhaps reflecting the heightened sensitivity of the brain during these developmental periods.[17,18]

Additionally, there is growing evidence that neurotoxic susceptibility may also differ by sex[19-21]. For example, in a study of Taiwanese residents over age 60 who in 1979 were exposed as adults to high levels of PCBs and dioxin-like compounds in contaminated cooking oil, an adverse association was observed between blood PCB concentrations and performance on tests of attention, visual memory, and learning ability among the exposed women but not among the exposed men.[22] Among individuals aged 70-84 years old in NHANES, women also demonstrated greater reductions in cognitive scores with higher dioxin-like PCB exposure than men[23]. These studies provide early epidemiological evidence that age and sex may be important modifiers of TCDD's effects on the human brain over the life-course.

The hypothesized mechanisms underlying these interactions between age, sex, and dioxin exposure center on endocrine disruption. For example, in the case of older women, TCDD-related neurotoxicity may result from the loss of estrogen's neuroprotective effects during and after menopause.[24] Many neurological processes are mediated by dopamine transport and regulation in the prefrontal cortex, a system that estrogen promotes.[25] The inhibition of central dopamine resulting from the natural decline of estrogen in older women may be exacerbated by the neurotoxic activity of toxicants on these same receptors and may interact to hasten onset of cognitive decline among highly exposed older women. However, the relationship between TCDD and dioxin-like chemicals on neuropsychological functioning warrants further investigation, particularly with respect to sensitive windows of development and sex differences.

IV.IV Dioxins and Fetal Programming

The fetal origins of disease hypothesis posits that the fetal response to intrauterine challenges such as nutrient restriction, maternal stress, or exposure to toxic chemicals is maladaptive to healthy development and contributes to later disease onset.[26] A handful of studies of sibling pairs discordantly exposed *in utero* to the 1944/45 Dutch Hunger Winter have provided compelling evidence to support this theory, suggesting that epigenetic changes related to exposure to famine *in utero* predispose offspring to chronic metabolic dysregulation later in life.[27,28] Designing epidemiologic studies of an adequately long duration poses a significant roadblock to thoroughly evaluating Barker's hypothesis in humans. Despite these challenges, the literature corroborating the fetal origins of disease has made large strides in the last decade, with a wide scope of

outcomes and exposures represented in the literature[29]. Efforts to refine and replicate these research studies will provide necessary depth to our understanding of fetal programming.

TCDD has been shown to cross the placenta [30,31] and is hypothesized to interfere with fetal development. Evidence from animal studies and a small number of epidemiological investigations suggest those exposed *in utero* may be even more susceptible to the effects of TCDD.[32-40] For example, prenatal exposure to TCDD and dioxin-like chemicals has been linked to altered immune function, glucose regulation, steroidogenesis, and dental development in humans and animals.[41] During the fetal period, the developing brain is particularly sensitive to toxic insult. Studies of mice and monkeys have linked gestational TCDD exposure to hyperactivity and impaired learning and memory[42-46]. In children exposed prenatally to dioxins mixtures, sex-specific patterns in neurobehavior, specifically pertaining to language development, attention, nonverbal intelligence, and play behavior, have also been documented [47-51]. Dioxin is proposed to interfere with fetal programming through mechanisms of endocrine disruption, possibly by altering the structural brain dimorphism established by the hypothalamic-pituitary-gonadal and thyroid axes during fetal brain development[52-54]. Evidence for this theory comes from animal models demonstrating that TCDD acts on the neuroendocrine pathways of the aryl hydrocarbon receptor (AhR), impacting pubertal development, behavior, cortical dominance, and sex-specific changes in learning and memory.[55] TCDD's neurotoxicity in the human fetus, however, is less clear[41] and the sex-specific effects of TCDD on human neural development remain to be characterized.

IV.V Genetic Susceptibility to Dioxin

In addition to specific windows of toxicological susceptibility, mounting evidence suggests that genetic factors further modify the toxicity of environmental exposures, making some people more sensitive to health effects than others[56,57]. Single nucleotide polymorphisms (SNPs) in *AHR* and genes in its regulatory pathway such as cytochrome P450s (CYP) and AhR-repressor (AHRR) have been linked to a variety of adverse health outcomes including cancer and disorders of reproduction and development[58-60], but few studies have had adequate variability in exposure distributions and large enough sample sizes for informative gene-dioxin investigations.

Few studies have examined these gene-environment interactions in relation to dioxins in humans and, to date, only one, in a Japanese birth cohort, has focused on prenatal TCDD exposures[61]. Understanding how the interplay between genetic and environmental factors *in utero* may induce adverse outcomes will further elucidate mechanisms underlying the fetal origins of adult disease.[62]

IV.VI The Seveso Accident

On Saturday, July 10, 1976 at 12:37pm, a 2,4,5-trichlorophenol chemical reactor exploded at the ICMESA factory in Meda, a town near Seveso, Italy and 25km north of Milan. As operators shut down the factory for the weekend, a reactor was inadvertently heated, and going unnoticed, triggered a runaway chain reaction overnight. The explosion released an aerosol cloud of sodium hydroxide, ethylene glycol, sodium trichlorophenate and nearly 30 kg of TCDD, a byproduct of the uncontrolled exothermic reactions. The cloud was dispersed over an 18 km^2 area [63], exposing local residents to high levels of TCDD in one of the worst industrial accidents on record.

In the days that followed, the affected area experienced high animal and plant mortality, particularly among rabbits and poultry. In the weeks that followed, residents exposed to the toxic plume presented with nausea, headaches, eye irritation, and hundreds of residents, mostly

children, developed chloracne, a blistering rash characteristic of acute dioxin poisoning [64,65]. As the toxic consequences came to light, residents were advised to avoid consumption of locally-sourced foods and thousands of feed animals were slaughtered as precaution. At the time, no biomarker existed for TCDD exposure but soil levels in the surrounding area were used to classify the contamination into zones A, B, and R. Zone A, in the immediate vicinity of the factory, had the highest soil levels and its 736 residents (212 families) were evacuated between July 26th and August 2nd. Though the topsoil in Zone A was later remediated, residents were permanently barred from returning. Their homes, the factory, and other structures were razed to ensure complete decontamination and protection of human health. The spreading alarm of the toxic after-effects among residents compelled the government to advise abortions for pregnant women in the area, a restricted practice only legal under special circumstances.

With so much unknown about the exposure and its implications for human health, Dr. Paolo Mocarelli and colleagues at the local Hospital of Desio who had been treating patients of the Seveso accident, began collecting blood specimens from 30,000 residents. With the foresight that TCDD would one day be measureable in blood, his team, supported by funding from the local government (Regione Lombardia), stored a portion of these samples (1-3mL) for future use. Follow-up of these residents continued annually from July 26, 1976 until June 30, 1982 to assess their health [66].

Given the limitations of dioxin exposure assessment in 1976, the delayed response to the accident, and a general lack of knowledge about TCDD at the time, many details about the route of exposure remain unknown. The Seveso residents closest to the explosion most likely received a combination of inhalation and dermal exposure to TCDD. Ingestion may have also occurred if locally-grown meat and produce were consumed. Before the advent of a biomarker in 1987 [67], zone of residence was used as a proxy for dioxin exposure in Seveso health studies. However, later exposure studies indicated that zone was only crudely correlated with internal exposure; TCDD blood levels varied widely within zone, likely reflecting inter-individual variability in behaviors and diet following the accident. Nevertheless, biomarker studies confirmed that Seveso residents had higher serum dioxin levels than the general population in Italy in the years immediately following the accident [68]

Dioxin's notoriety was further consolidated in the late 1970s by two highly visible events 1) massive industrial pollution in the Love Canal disaster, spurring the passage of the Superfund Cleanup program, and 2) mounting concern over dioxin contamination in Agent Orange, a defoliant widely used during the Vietnam War. Under these historical pressures, the Centers for Disease Control and Prevention (CDC) were able to develop by 1987 a high-resolution gas chromatography/high-resolution mass spectrometry analytical method to measure TCDD concentration in human serum [67]. This advance enabled more rigorous epidemiology of the health of effects of TCDD and paved the way for initiation of the Seveso Women's Health Study (SWHS) in 1996, led by Dr. Brenda Eskenazi at the University of California, Berkeley. This cohort of 981 women who were newborn to 40 years old at the time of the accident, was unique in not only being the largest study of the health of dioxin's effects in a female population but also had the benefit of being the only study at the time with TCDD exposure measured in blood taken close to the time of the exposure. The SWHS, with funding from the National Institutes of Health (NIH), completed two subsequent follow-ups in 2008-09 and 2014-16 and is ongoing. In 2014-16, the study began enrolling children born to SWHS women after the accident into the Seveso Second Generation Health Study.

IV.VIII Significance

Despite over thirty years of toxicological study, TCDD's mechanism of action in the human body, including the factors that shape susceptibility and variability in the human response, remains poorly understood.[6] The evidence in animals consistently indicates TCDD's neurotoxicity but few studies have investigated its effects on the human brain. Furthermore, few investigations have had the ability to disentangle the effects of TCDD from other related and often correlated exposures of PCBs, furans, and other dioxins. One of the most important aspects of this study is the unique exposure scenario in which the mothers were exposed directly, but the children have been exposed predominately through *in utero* and lactational transfer from the mother. Thus, this population provides the first opportunity to examine *in utero* exposure to TCDD and health of the second generation largely unconfounded by continued exposure during childhood. In addition, the Seveso population's acute exposure experience, measured in blood samples taken close to the time of the accident, allows an examination of TCDD in near isolation from environmental co-exposures, typically unattainable in other study populations. Insight into windows of susceptibility and genetics of TCDD toxicity may be widely applicable to toxicological mechanisms of PCBs, PAHs, and other organochlorine compounds and may identify susceptible groups for future targeted interventions.

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1. Neurocognitive and physical functioning in the Seveso Women's Health Study

1.1 ABSTRACT

Background: 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is neurotoxic in animals but few studies have investigated its effects on the human brain. Related dioxin-like compounds have been linked to poorer cognitive and motor function in older adults, with effects more pronounced in women, perhaps due to the loss of neuro-protective estrogen in menopause. On 10 July 1976, a chemical explosion in Seveso, Italy, resulted in one of the highest known residential exposures to TCDD. In 1996, we initiated the Seveso Women's Health Study, a retrospective cohort study of the health of the women who were newborn to 40 years old in 1976. Here, we investigate whether TCDD exposure is associated with physical functioning and working memory more than 20 years later. Methods: Individual TCDD concentration (ppt) was measured in archived serum collected soon after the explosion. In 1996 and 2008, we measured physical functioning (n=154) and working memory (n=459), respectively. We examined associations between serum TCDD and motor and cognitive outcomes with multivariate linear regression and semi-parametric estimators. Results: A 10-fold increase in serum TCDD was not associated with walking speed (adjusted β=0.0006 ft/sec, 95% Confidence Interval (CI): -0.13, 0.13), upper body mobility (adjusted β=-0.06, 95% CI: -0.36, 0.23), or manual dexterity (adjusted β=0.34, 95% CI: -0.65, 1.33). We observed an inverted U-shaped association in grip strength, with poorer strength in the lowest and highest TCDD exposure levels. There was no association between TCDD and the Wechsler digit and spatial span tests. Neither menopause status at assessment nor developmental timing of exposure modified associations between TCDD and working memory. Conclusions: Our findings, in one of the only studies of TCDD's effects on neuropsychological and physical functioning in women, do not indicate an adverse effect on these domains, with the exception of a U-shaped relationship with grip strength. Given the limited assessment and relative youth of the women at this follow-up, future work examining additional neuropsychological outcomes is warranted.

1.2 INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a global environmental pollutant released into the environment through industrial sources of combustion. Due to its exceptional toxicological potency and chemical stability, TCDD ranks among the 2001 Stockholm Convention's "dirty dozen" of ubiquitous persistent organic pollutants (POPs) [1]. TCDD exerts its biological toxicity primarily through its binding affinity for the aryl hydrocarbon receptor (AhR), a nuclear receptor and transcription factor that regulates myriad biological processes related to development, cell growth, apoptosis, and immune function [2]. TCDD is a member of a wider class of halogenated aromatic compounds such as polychlorinated dibenzo-*para*-dioxins (PCDDs), dibenzofurans (PCDFs), and certain polychlorinated biphenyls (PCBs) that share this mechanism of action via the AhR. Dioxins bioaccumulate in adipose tissue [3] and have a long half-life of 4 to 11 years in the human body [4,5]. While levels of dioxins found in humans have decreased substantially over the last few decades, aging populations with high fish and meat consumption are particularly vulnerable to accruing higher, potentially toxic body burdens over the life course [6].

In vitro studies demonstrate that TCDD-induced activation of the AhR, through altering endocrine function and expression of genes related to apoptosis and oxidative stress, promotes premature cell senescence in rat and human neurons and animal studies have reported impairments in memory, spatial and visual learning, and fear response with developmental exposure to TCDD and dioxin-like compounds [7-13]. However, few studies have investigated the neurotoxic effects of TCDD in humans. Studies of U.S. veterans exposed to TCDD through the spraying of Agent Orange during the Vietnam War have found that men with the highest dioxin exposures performed poorly on tests of motor coordination and verbal memory compared to their unexposed peers [14,15]. Neuropathic signs, writer's dystonia, and tremor have also been documented in small studies of individuals exposed to TCDD occupationally and during the Seveso accident [16-19]. Several studies in general populations with exposures closer to background levels have found associations between dioxin-like PCB body burdens and impairments in motor function, memory, learning, and executive function [20-23].

Susceptibility to the neurotoxic effects of dioxin may vary by sex and age. For example, an inverse association was observed between blood PCB concentrations and performance on tests of attention, visual memory, and learning ability among older Taiwanese women but not among men who were exposed as adults in 1979 to high levels of PCBs and dioxin-like compounds in contaminated cooking oil (the Yucheng cohort) [24]. Similarly, a study of older adults in NHANES, where exposures were closer to background, found adverse associations between dioxin-like PCB serum concentrations and poorer cognitive scores, with the association most pronounced among women aged 70+ years [25]. An excess of Parkinson's disease, dementia, and amyotrophic lateral sclerosis was also observed among women occupationally exposed to PCBs [26]. Given the wide cross-talk of the AhR with several hormonal pathways, the mechanism underlying these interactions between age, sex, and exposure to dioxin-like compounds may be exacerbated by the loss of estrogen's neuroprotective effects during and after menopause [27]. Estrogen-related loss of brain dopamine could also contribute to lowered physical functioning and reductions in muscle mass and strength following menopause [28].

In the present study, we investigated the neurotoxic effects of dioxin in the Seveso Women's Health Study (SWHS), a historical cohort study of women residing around Seveso, Italy at the time of an industrial accident on July 10, 1976 that resulted in one of the highest levels of residential TCDD contamination known [29]. We hypothesized that higher 1976 serum concentrations of dioxin would be inversely associated with physical and cognitive functioning and that adverse associations would be most pronounced in postmenopausal women. In addition to susceptibility factors at the time of assessment, we also considered differences in susceptibility among those exposed at younger ages, while the brain, particularly areas related to working memory such as the prefrontal cortex, are still developing [30]. The study of neurodevelopmental effects of dioxin have largely focused on the perinatal period but the continued susceptibility of the brain to environmental toxicants during its rapid growth, neuronal pruning, and maturation during childhood and young adulthood (up to about 25 years of age) is not well understood [31,32].

1.3 MATERIALS AND METHODS

Study population

Recruitment of the SWHS cohort has been described previously [33]. Briefly, this historical cohort study recruited eligible women who were newborn to 40 years of age on July 10, 1976, resided at that time in the highest contaminated areas (Zones A and B), and had adequate stored

serum for analysis of TCDD collected soon after the explosion. Enrollment took place from March 1996 to July 1998, and 981 women (80% of those eligible) participated. A subset of the oldest women (31-40 years in 1976) who were interviewed after September 1997 (n=173 of 229) were invited to participate in an assessment of physical functioning added as part of the study visit. Of those invited, 19 women refused to participate in any of the tests, leaving 154 women (89% of eligible) who completed the physical function tests.

Between April 2008 and December 2009, we conducted a follow-up of the SWHS cohort: 833 (85%) of the original 981 women could be contacted and agreed to participate (16 were deceased and 36 could not be located). Data collection was already underway when findings of lowered working memory and other neuropsychological measures in the Yucheng cohort were published [24]. This motivated development of an ad-hoc assessment of neuropsychological outcomes in the SWHS. Starting in December 2008, partway through the 2008-2009 follow-up of the cohort, remaining participants (n=459) were invited to complete an assessment of working memory as part of the study visit. We excluded two with Turner's syndrome leaving 457 participants (sample flowchart presented in Supplementary Figure 1.1).

Procedure

The study was approved by the Institutional Review Boards of the participating institutions and written informed consent was obtained from all women prior to participation. Details of the study procedure for the 1996 and 2008 studies are described elsewhere [33,34]. In both 1996 and 2008, information on covariates such as demographic and lifestyle factors and medical history were obtained from a questionnaire administered in private by a trained nurse-interviewer and followed by a brief medical exam which included anthropometric and blood pressure measurements. Interviewers were blinded to participants' serum TCDD levels and zones of residence.

Laboratory Analyses

Archived serum samples collected in 1976 were stored at -20ºC until shipped to the Centers for Disease Control and Prevention (CDC) for analysis in 1998. TCDD was measured in archived sera by high-resolution gas chromatography/high-resolution mass spectrometry methods [35]. Prior to statistical analysis, serum TCDD levels were adjusted for blood lipid concentrations by dividing TCDD on a whole-weight basis by total serum lipid content, estimated from measurements of triglycerides and total cholesterol [36]. Serum TCDD levels were reported in picograms per gram lipid or parts per trillion (ppt). The median serum sample weight for these samples was 0.65 g, and the median lipid-adjusted limit of detection was 18.8 ppt. Samples below the limit of detection (LOD) (9.4% in full cohort) were assigned a value equal to one-half of the LOD, an approach supported by Hornung and Reed's comparison study of multiple estimation methods for non-detectable samples[37]. Details of the serum sample selection and TCDD concentrations measured in 1976 serum are presented elsewhere [33,38].

Physical function assessment in 1996

The physical function assessment administered in 1996 included four validated physical tasks chosen for their ease of implementation, reliability, and frequent use in studies of community-dwelling older adults [39]: 1) a 10-foot walking test of functional mobility, 2) a coin-flipping test of manual dexterity, 3) a grip strength test, and 4) a reach down test of lower body mobility [40]. Together, these tests represented a diverse cross-section of physical performance.

For the 10-foot walking test, participants were asked to walk back and forth on a 10-foot long course for two minutes at their regular speed as if walking down the street to go to the store. We used the number of lengths walked in this time to calculate the average walking speed (ft/s). For the coin flip test, participants were timed on how quickly they could turn five 50 lire coins from the heads to tails position on a table without dropping them. For the grip strength test, participants, while in the standing position, were asked to squeeze a dynamometer three times in each hand. We analyzed grip strength in two ways: the average of the three measures and the highest of the three measures for the dominant and non-dominant hands. Lastly, in the reach down test, participants were timed (in seconds) on how quickly they could, from a standing position, reach down to pick a pen off the floor and return to standing.

Working memory assessment in 2008

The neurocognitive assessment in 2008 included the Wechsler Adult Intelligence Scale (WAIS) digit span and spatial span tests [40]. The digit span test targets verbal working memory and engages executive function skills of attention, associability by linking items so as to better recall them, and mathematical ability; the backward task requires more complex storage and retrieval. The forward and backward subtests begin with the administrator saying aloud 2 digits (i.e. 1-7) and progresses until criterion up to 8 digits. The participant is then asked to retrieve and verbalize the span back to the administrator in either the forward or backward order and must successfully repeat two lists at each sequence length before another digit is added. The digit span (forward, backward) is the length of the longest sequence recalled correctly (maximum of 8 forward and 7 backward). In a normative sample of Italian adults (aged 30-70 years) the mean (±SD) of the forward and backward digit spans was 6.0 (±1.0) and 4.7 (±1.1), respectively [41].

The spatial span test, an adaptation of the Corsi block-tapping test [40], is considered a visual-spatial analog of the digit span. The task assesses an individual's ability to remember a 3-dimensional sequence of tapping on a grid of white squares by the administrator immediately after their presentation. The spatial span (forward, backward) is the length of the longest sequence correctly recalled with a maximum of 8. The normative mean (±SD) forward and backward spatial spans in Italian adults is 5.5 (±1.0) and 4.9 (±1.0), respectively [41].

Each of the four subtests was repeated twice, and each subtest raw score was calculated as the sum of the two trials. The maximum raw score was 14 for the backward digit span subtest and 16 for the other three subtests (forward digit span, forward and backward spatial span).

Statistical analysis

Because the serum TCDD distribution was approximately log-normal, TCDD levels were $\log_{10}$-transformed. Serum TCDD was analyzed as both a continuous exposure ($\log_{10}$TCDD) and categorized to four levels. In the categorical analysis, TCDD levels ≤20ppt, which were comparable to background serum levels of unexposed Italian women in 1976, served as the reference group [42,43]; the remaining three exposure categories were defined by exposure tertiles calculated across the full cohort, producing groups of ≤ 20, 20.1–47.0, 47.1–135.0, and > 135 ppt.

Potential confounding variables were chosen *a priori* based on the literature of human neurotoxicity of PCBs/dioxins and earlier work in the SWHS. We considered: educational attainment, smoking, alcohol consumption, age at interview, age at explosion, menarche status at explosion, menopause status (pre-/post-) at interview (>12 months without a menstrual cycle or surgical menopause), body mass index category [BMI; kilograms per meter squared categorized

as underweight/normal (< 25 kg/m^2), overweight BMI ≥ 25 kg/m^2 and < 30 kg/m^2) and obese (BMI ≥ 30 kg/m^2)], , and marital status. We used a directed acyclic graph (DAG) of the assumed underlying causal relationships among variables to inform covariate selection into the initial adjusted model (Supplementary Figure 1.2) and then, following a change-in-estimate approach, further pared down the adjustment set to contain covariates changing the association between TCDD and the outcome by more than 10% [44].

The physical function and working memory outcomes were initially all considered as continuous variables. We examined the functional form of the relationship between each of the outcomes and TCDD using locally weighted scatterplot smoothing (lowess) and restricted cubic splines, and compared regression models with and without a squared term on TCDD with a likelihood ratio test [45]. If a linear model appeared adequate, we used multivariate linear regression to assess the dose-response relationship between dioxin and the physical and cognitive function tests, conditional on confounders. In sensitivity analyses, we modeled TCDD categorically and obtained marginal estimates of effect using semi-parametric estimation [targeted maximum likelihood estimation (tmle) implementing Superlearner] that allowed us to make fewer assumptions about the functional form, thus mitigating any bias in the effect measure introduced by mis-specified parametric modeling [46-48]. For regression models, variances were estimated using a robust sandwich estimator [49] with the exception of TMLE which was based on the influence curve.

For the working memory analysis, we considered effect modification by developmental status at exposure including menarche status in 1976 (premenarche versus postmenarche) and before/after peak brain development (< age 25 versus ≥ age 25) [50,51], and menopause status at assessment (premenopause vs. postmenopause). (All women assessed on physical functioning were post-menarche at the time of the explosion and post-menopause at assessment.) Effect modification was modeled by creating a cross-product term between $\log_{10}$TCDD and the effect modifier of interest. Interactions were considered significant if the p-value for the cross-product term was < 0.2.

In sensitivity analysis, to account for potential selection bias due to subsampling of women within each wave and for loss-to-follow-up across the 1996 and 2008 waves, we re-estimated the parameters with inverse-probability weights of censoring [52]. The censoring weights were fit using a library of 5 algorithms in Superlearner (SL.glm, SL.gam, SL.glmnet, SL.mean, SL.randomForest) and stabilized based on the proportion of women included in the present analysis[48].

1.4 RESULTS

Participant characteristics

Descriptions of the 1996 physical function and 2008 working memory study samples in comparison to the full SWHS cohort are provided in Table 1.1. The 1996 physical function sample was older than the full SWHS cohort but did not differ with respect to other sociodemographic characteristics; the 2008 working memory sample was of similar age to the full SWHS cohort and was also similar in sociodemographic characteristics (see Table 1.1). In the 1996 assessment of physical functioning, the average age at interview was 57.3 (SD± 2.9) years and all women were postmenopausal. Almost all were married (97%) and reported the household primary wage earner had completed middle school, the required education (96%), 22% reported ever smoking, and 49% were regular alcohol drinkers. In the 2008 working memory sample, 53% of the women were postmenopausal and averaged 52.3 (SD± 11.3) years

at the time of the memory assessment. The majority were married (91%) and reported the primary wage earner had completed the required education (65%), and 38% and 37% had a history of smoking and regular alcohol consumption, respectively. In addition, 30% were pre-menarche at the time of the explosion.

The median 1976 TCDD serum concentration was 45.2 ppt [interquartile range (IQR) = 28 – 100] for women who completed the physical function assessment and 60.1 ppt (IQR = 29 – 150) for women who completed the working memory assessment compared to a median of 55.9 ppt (IQR = 28 – 157) for the full SWHS cohort. Among women who were pre- and post-menopausal at the time of the working memory assessment, the median levels were 74.3 ppt (IQR = 33 – 207) and 46.3 ppt (IQR = 25 – 103), respectively. Women who were pre-menarche at the time of the explosion had higher serum TCDD levels (median = 141.2 ppt; IQR = 48 – 265) than those who were postmenarche (median= 45.6 ppt; IQR = 25 – 93). These associations between exposure levels and age have been documented previously [43].

The distribution of the neurophysiological function scores in 1996 and 2008 are presented in Table 1.2. Physical function and working memory scores were normally distributed with the exception of the reach down test where the majority (94%) of participants completed the task within the narrow range of 1–3 seconds while the rest of the distribution was positively skewed toward longer test times.

TCDD and physical functioning

The linear models for the physical function outcomes are presented in Table 1.3. We observed no association between a 10-fold increase in 1976 TCDD serum concentrations and average walking speed (adjusted $\beta = 0.0006$; 95% Confidence Interval (CI): -0.13, 0.13), manual dexterity (coin flip)(adjusted $\beta = 0.32$, 95% CI: -0.65, 1.33), and lower body flexibility (reach down test)(adjusted $\beta = -0.04$; 95% CI: -0.36, 0.23) in the postmenopausal women twenty years after the accident, after adjusting for age at assessment, primary wage earner's highest education level (i.e. spouse for majority of participants), and BMI in 1996.

The relationship between $\log_{10}$TCDD and dominant and non-dominant hand grip strengths showed evidence of non-linearity. In separate quadratic models for average grip strength in dominant and non-dominant hands, the first and second order $\log_{10}$TCDD terms were statistically significant. In these two models, the first order $\log_{10}$TCDD terms were positive and the quadratic terms were negative, suggesting an inverted U-shape curve at which grip strength improved at low levels of TCDD but decreased with higher exposure levels (see Figure 1). This pattern was also supported by models using a restricted cubic spline function with 4 knots. Models examining the highest grip strength yielded similar findings (Table 1.3). Fifty-five women were unable to perform the grip strength test due to self-report of arthritis (n=47), tendonitis (n=6), or recent hand/arm surgery (n=2); though the proportion with arthritis/hand pain was higher than the typical prevalence of osteoarthritis in Italian women at this age [53], these women did not differ significantly from those who did perform the test with respect to age or TCDD levels. Modeling all of the physical functioning outcomes as a function of categorized TCDD levels based on the distribution of exposure in the full cohort (Supplementary Table 1.1) provided similar inference as the continuous models.

In sensitivity analyses, when we adjusted for loss-to-follow up with inverse-probability weights results were similar (data not shown).

TCDD and working memory

Adjusting for age at assessment and primary wage earner's highest education level, a 10-fold increase in 1976 serum TCDD levels was not associated with verbal or spatial working memory measured about thirty years after the accident (Table 1.4) and there was no evidence of non-linearity in the associations. We found some evidence of effect modification by menarche status in 1976 (Table 1.4). A 10-fold increase in serum TCDD was associated with a better forward digit span score (adjusted $\beta = 0.42$; 95% CI: 0.03, 0.81) among women who were post-menarche in 1976, but not among those who were premenarche in 1976 (adjusted $\beta = -0.05$; 95% CI: -0.43, 0.32) (p-interaction = 0.06). We found no evidence of effect modification of the association between continuous TCDD and memory scores by exposure before or after peak brain development (age 25) (Supplementary Table 1.2) or by menopause status at study visit (Supplementary Table 3).

In sensitivity analyses, we examined the consistency of the working memory association when the assumptions of parametric models were relaxed with use of targeted maximum likelihood estimation (tmle) using SuperLearner (Supplementary Table 1.4). These models produced similar findings to the linear regression models using categorical exposure, suggesting no association between TCDD exposure and working memory (Supplementary Table 1.5). Further, results were robust in models including inverse-probability of censoring weights to account for selection bias in subsampling and loss-to-follow up in the cohort (data not shown). We also did not observe evidence of effect measure modification by exposure before menarche or during peak brain development at the explosion, when TCDD was categorized (data not shown).

1.5 DISCUSSION

In our study, we examined physical and cognitive function in women several decades after exposure to dioxin released in an industrial explosion in Seveso, Italy. We found no significant associations between their 1976 serum TCDD levels and several measures of motor function and working memory (digit span or spatial span) approximately twenty and thirty years after exposure, respectively. Average and highest grip strengths in the dominant and non-dominant hands were the only endpoints with a suggestive non-monotonic relationship characterized by diminished strength at the lowest and highest levels of dioxin exposure. At present, we are unaware of a biological explanation for the shape of this relationship and caution it may be a spurious result from a small sample. This finding warrants confirmation although few other populations will have exposure as high as the Seveso cohort.

In 1976 at the time of the explosion, the women in the physical function study were in their 30s – an age window not typically considered a critical period of brain development [54,55]. Further, the women were assessed for motor ability in their 50s and 60s when such functions are beginning to decline; though our findings suggest that TCDD may not accelerate this course in the short term, an investigation at later life stages, when physical function is in steeper decline, may better reveal dioxin's potential to alter the aging process. Further, we did not assess the physical function of the younger women in the cohort (less than 30 years at the time of the explosion); as they age, these outcomes may warrant another look. Our findings are consistent with a cross-sectional study of older Michigan residents with diets high in PCB-contaminated fish in which no association was found between PCB exposure and hand steadiness nor visual-motor coordination in models adjusted for age and gender [56]. However, it is important to note that the physical function tests we examined are reflective of general physical performance and are not typically as sensitive as the tests of finer neuromotor changes in the Michigan study.

Our findings on working memory are not consistent with those reported by Lin et al. in the Yucheng cohort, which was exposed primarily to a mixture of PCBs and furans. They reported a dose-response relationship between exposure and poorer performance on learning and memory tests, including the digit span test, in 313 exposed women 25 years after the accidental rice oil poisoning [24]. This discrepancy could be attributable to the older age of their sample (mean = 69.5 (±5.9) years versus 52.3 (±11.3) years in our sample), differences in the mix of compounds the two populations were exposed to, or perhaps differences in the populations' genetic susceptibilities. Though our study examined cognitive susceptibility around the time of menopause, several studies of PCB/dioxins in older adults have reported cognitive reductions in women over the age of 70, suggesting that the reserve capacity defending the brain from neurotoxins may diminish with aging [21,25,57]. We note that the oldest subject in our analysis was 73 years old with fewer than 10% of the sample aged 70 years or more. Thus, any age-related susceptibility to dioxin neurotoxicity may not manifest nor be readily measureable until the cohort shifts toward elderly (as we also previously noted with the physical function tests).

Another potential explanation for the differences between our findings and those in the Yucheng cohort is the comprehensiveness of the latter's neuropsychological assessment. While they found significant poorer performance on tests of forward and backward digit span in women with increased exposure to PCB/PCDFs, they also reported dose-response inverse relationships with additional measures of attention, verbal memory, psychomotor function, visual scanning, and learning ability that we were not able to assess in the SWHS [24]. We only examined working memory and physical functioning in the SWHS, and thus the effects of TCDD exposure on additional neurophysiological domains cannot be gleaned from this study and remain unknown. Future work should consider a more rigorous and broad examination of neuropsychological function and include, for example, a refined battery of neuromotor tests and the digit symbol coding test which was sensitive to dioxin-like chemicals in two different studies [24,25].

With measurements of working memory in 2008, we were able to examine dioxin's neurotoxic potential across two developmental windows in the female life course – menarche and menopause. Gonadal hormones are integral to brain development, transitioning from organizational effects on brain differentiation in early life to activational effects of neural plasticity and behavioral functions throughout adulthood [58]. Thus, we expected initial TCDD exposure and subsequent body burdens in relation to these periods of neuroendocrine regulation could have important and disparate effects on the aging brains of women who were different ages, ranging from 0-40 years, at the time of the accident. However, in our analysis, we found that dioxin exposure at younger ages, either during the window of peak brain development before age 25 or before menarche in adolescence, was not associated with cognitive performance thirty years later.

We previously reported a non-monotonic relationship between TCDD and risk of earlier menopause in the SWHS, consistent with the hypothesized effects of endocrine-disrupting chemicals [59]. We expected that the hormonal declines of menopause could also modify a woman's later life susceptibility to dioxin neurotoxicity since many women experience temporary neurological impairment, particularly related to memory, during perimenopause though the longevity of this effect is unclear [60,61]. The inhibition of central dopamine resulting from the menopause-related decline of estrogen in older women as well as age-related loss of dopaminergic neurons, may be exacerbated by the neurotoxic activity of dioxin-like compounds on the dopamine system to hasten cognitive decline among highly exposed older women [12,58,62]. Toxicologic studies *in vitro* have suggested that TCDD, in addition to disrupting regulation of

the neuroendocrine system, can alter neuronal biochemistry, inhibiting calcium uptake and neurotransmitter synthesis and signaling [63-67]. However, the physiologic implications of these molecular changes have not been closely studied in adult animals or humans. In this study, we did not see evidence of modified sensitivity to TCDD neurotoxicity among menopausal women, the majority of whom were adults at the time of explosion.

TCDD has been shown to cross the placenta and is hypothesized to interfere with fetal development in both humans and animals [68,69]. Animal studies and limited epidemiological evidence suggest those exposed *in utero* may be even more susceptible to the effects of TCDD [70-78]. TCDD's neurotoxicity in the human fetus, however, is less clear and the sex-specific effects of TCDD on human neural development remain to be characterized [79]. In future analyses, we will examine the relationship of *in utero* TCDD exposure on the children born to the women in the SWHS.

The present study has several strengths. First, the SWHS cohort is one of the only studies of sufficient size and wide exposure variability to TCDD with background levels of exposure to other dioxin-like compounds [80] to examine the effects of dioxin on women's health. Further, the study utilizes direct measurements of serum TCDD close to the time of the accident and a prospective study design.

In addition to the limited scope of the study's neuropsychological assessment and the possibility that the window of the present study is too premature to observe the age-related neurotoxicity of TCDD, the small sample size of the physical function analysis raises concerns about power and selection bias. Further, a third of the women who completed the physical function assessment did not participate in the grip strength test due to hand pain or surgery. Since these women did not differ significantly in TCDD levels and age from those who completed the test, selection bias is unlikely to explain the grip strength finding. Nevertheless, the proportion of women refusing the test due to arthritis/hand pain is in excess of the expected prevalence in older Italian women (18%), further qualifying these findings. Our data in the working memory analysis lack a large cell size of younger people with lower TCDD levels (and older individuals with high exposure), leaving open the possibility of residual confounding by age. While we were only able to collect outcome data on a subsample of eligible women in both 1996 and 2008, sensitivity analyses exploring the possibility of selection bias with inverse probability of treatment weighting did not appreciably change the results. Nevertheless, the background exposures of 1976 are substantially higher than background levels today (around 2ppt), which makes our lowest exposed individuals fairly exposed by today's standards, and thereby limits our ability to generalize the relationship to these lower levels.

1.6 CONCLUSIONS

In summary, we did not see evidence of an adverse relationship between TCDD exposure and long-term effects on working memory in a cohort of women exposed postnatally to high levels of dioxin released during the Seveso accident. TCDD exposure does not appear to be associated with physical functioning in the oldest women in the cohort, possibly with the exception of grip strength where we observed an inverted U-shaped association. However, the cohort was still relatively young at the age of assessment and continued follow-up of their neurophysiological health into old age, with a more comprehensive neurocognitive assessment, is warranted. The interplay of past and current environmental exposures on the aging brain is understudied and of greater importance given the increasing longevity of populations in industrialized countries.

1.7 ACKNOWLEDGEMENTS

This study was supported by grants F06 TW02075-01 from the National Institutes of Health, R01 ES07171 and 2P30-ESO01896-17 from the National Institute of Environmental Health Sciences, R82471 from the U.S. Environmental Protection Agency, and 2896 from Regione Lombardia and Fondazione Lombardia Ambiente, Milan, Italy.

We acknowledge the significant contributions in sample analyses made by D. Patterson, L. Needham, and W. Turner to the Seveso Women's Health Study. We gratefully acknowledge A. Parigi for coordinating data collection at Hospital of Desio and A. Wesselink for assistance in data management. We also dedicate this work to W.A. Satariano, a leader in the field of aging and health and a beloved colleague and friend. He passed away in May 2017 and is greatly missed.

1.8 TABLES

Tables 1.1-1.4 Neurocognitive and physical functioning in the SWHS

Table 1.1 – Select characteristics of participants in complete cohort and in physical function and working memory assessments, SWHS, Italy, 1996-2009 [n(%)]

Characteristic	SWHS Full Cohort *	1996 Physical Function Subgroup	2008 Working Memory Subgroup
Total	n=981	n=154	n=459
Characteristics at explosion			
Age at explosion, years			
0-10	232 (23.7)	0	111 (24.2)
11-20	279 (28.4)	0	144 (31.4)
21-30	241 (24.6)	1 (0.7)	105 (22.9)
31-40	229 (23.2)	153 (99.3)	99 (21.6)
Menarche status at explosion			
Premenarche	284 (28.9)	0	139 (30.2)
Postmenarche	69 (71.1)	154 (100)	320 (69.8)
Exposure before age 25			
<25 years	613 (62.5)	0	301 (65.6)
≥25 years	368 (37.5)	154 (100)	158 (34.4)
Smoking status			
Never	827 (84.3)	126 (80.8)	387 (84.3)
Ever	154 (15.7)	28 (18.2)	72 (15.7)
Alcohol status			
Never	772 (78.8)	85 (55.2)	367 (80.0)
Ever	209 (21.3)	69 (44.8)	92 (20.0)
Serum TCDD, ppt (median (IQR))	55.9 (28, 157)	45.2 (28,100)	60.1 (29, 150)
Characteristics at follow-up			
Age at interview, years (Mean±SD)	NA	57.3 ± 2.9	52.3 ± 11.3
Menopause status			
Premenopausal	484 (49.3)	0	216 (47.3)
Postmenopausal	496 (50.6)	154 (100)	241 (52.7)
Primary wage earner's education			
≤ Middle School	627 (63.9)	148 (96.1)	297 (64.7)
≥High school	354 (36.1)	6 (3.9)	162 (35.3)
Marital Status			
Never	76 (7.8)	4 (2.6)	43 (9.4)
Ever	905 (92.3)	150 (97.4)	416 (90.6)
Smoking status			
Never	619 (63.1)	120 (77.9)	286 (62.3)
Former	194 (19.8)	11 (7.1)	105 (22.9)
Current	168 (17.1)	23 (14.9)	68 (14.8)

Table 1.1 (cont.)

Characteristic	SWHS Full Cohort *	1996 Physical Function Subgroup	2008 Working Memory Subgroup
Alcohol status			
Never	618 (63)	78 (50.6)	288 (62.7)
Former	44 (4.5)	14 (9.1)	16 (3.5)
Current	319 (32.5)	76 (49.4)	155 (33.8)
BMI Category			
Underweight	26 (2.6)	1 (0.7)	8 (1.7)
Normal	437 (44.6)	71 (46.1)	200 (43.6)
Overweight	302 (30.8)	57 (37.0)	142 (30.9)
Obese	216 (22.0)	25 (16.2)	109 (23.8)

* Last follow-up information on full cohort obtained from 1996 data for women who did not participate in 2008 follow-up.

Table 1.2 – Summary of measures (mean ± SD) of physical functioning and working memory tests, Seveso Women's Health Study, Italy 1996-2009

Measurement	**n**	**mean ± SD**	**median**	**min**	**max**
Coin Flipping (sec)	153	7.88 ± 2.43	7	3	19
Walking Speed (ft/sec)	149	2.12 ± 0.36	2.08	1.25	2.22
Reach Down Test (sec)	152	2.19 ± 1.50	2	1	16
Grip Strength					
Dominant (N)	98	23.73 ± 4.63	23.67	11	34.67
Non-Dominant (N)	98	22.52 ± 4.87	22.67	6	34.33
Digit Span					
Forward	459	8.03 ± 1.96	8	3	14
Backward	459	4.53 ± 1.98	4	0	12
Spatial Span					
Forward	459	6.94 ± 1.73	7	2	13
Backward	459	5.88 ± 2.03	6	0	11

Table 1.3 – Multivariable linear regression analyses for the relationship of serum TCDD (log_{10}) with measures of physical functioning, Seveso Women's Health Study, Italy, 1996-97

Outcome	n	log_{10}TCDD (ppt) Adj.β[a] (95% CI)	$log_{10}TCDD^2$ (ppt) Adj.β[a] (95% CI)
Time to flip 5 coins (sec)	153	0.34 (-0.65, 1.33)	-
Walking speed (ft/sec)	148	0.0006 (-0.13, 0.13)	-
Reach down test time (sec)	152	-0.06 (-0.36, 0.23)	-
Average grip strength dominant hand (kg)	98	8.50 (0.78, 16.22)*	-2.06 (-3.69, -0.43)*
Average grip strength non-dominant hand (kg)	98	12.83 (5.61, 20.05)*	-3.15 (-4.66, -1.64)*
Highest grip strength dominant hand (kg)	98	9.48 (1.59, 17.37)*	-2.26 (-3.90, -0.63)*
Highest grip strength non-dominant hand (kg)	98	12.59 (5.39, 19.80)*	-3.11 (-4.61, -1.60)*

[a]Adjusted for age at interview, primary wage earner education in 1996, BMI in 1996

* $p<0.05$

Table 1.4 – Multivariable linear regression analyses for the relationship of serum TCDD (log10) with measures of working memory span, stratified by menarche status at explosion, Seveso Women's Health Study, Italy, 2008–2009.

Outcome	**All women (n=457)** Adj.β[a] (95% CI)	**Pre-menarche in 1976 (n=139)** Adj.β[a] (95% CI)	**Post-menarche in 1976 (n=318)** Adj.β[a] (95% CI)	P_{int}
Digit Span				
Forward	0.18 (-0.09, 0.45)	-0.05 (-0.43, 0.32)	0.42 (0.03, 0.81)*	0.06
Backward	0.06 (-0.22, 0.34)	-0.1 (-0.51, 0.32)	0.17 (-0.23, 0.57)	0.52
Spatial Span				
Forward	0.05 (-0.21, 0.31)	0.17 (-0.28, 0.61)	0.04 (-0.32, 0.39)	0.47
Backward	-0.03 (-0.30, 0.24)	-0.17 (-0.66, 0.32)	0.06 (-0.31, 0.43)	0.54

[a]Models adjusted for age at interview, primary wage earner education in 2008

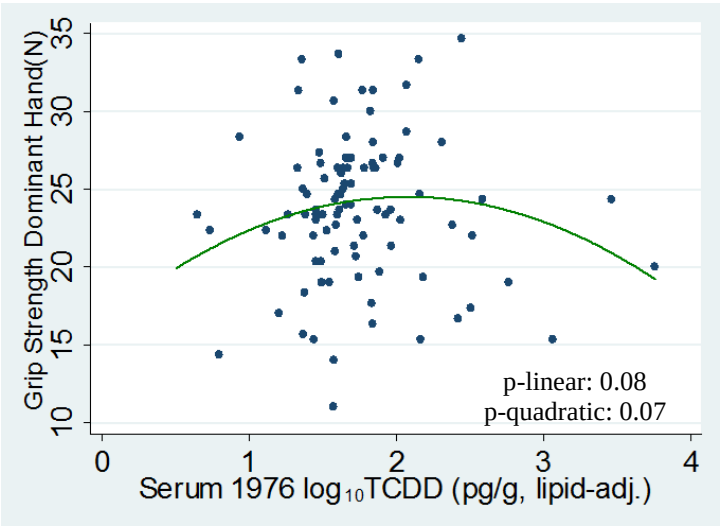
* $p<0.05$

1.9 FIGURES

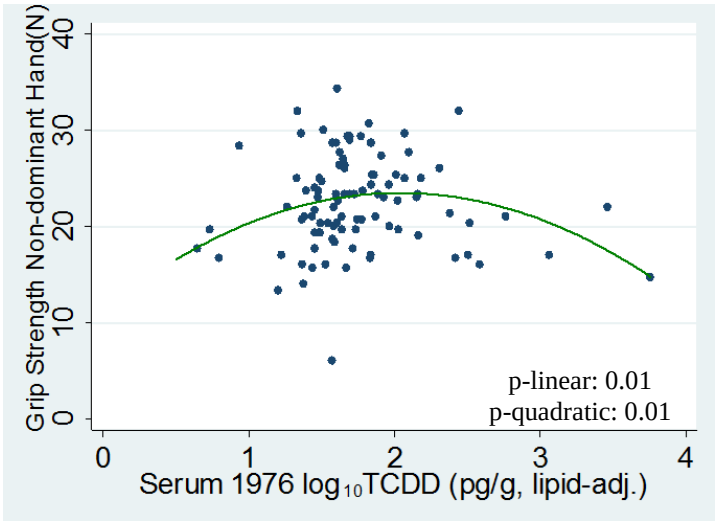
Figure 1.1 Neurocognitive and physical functioning in the SWHS

Figure 1.1– Plots of grip strength measurements for a 10-fold increase in 1976 levels of serum TCDD (ppt) in A) dominant hand and B) non-dominant hand. Green line shows fit of predicted values from quadratic model adjusted for age at assessment, BMI, and primary wage earner's education.

A



B



1.10 SUPPLEMENTARY MATERIALS

1.10.1 Supplementary Tables

Tables S1.1-S1.5 Neurocognitive and physical functioning in the SWHS

Supplementary Table 1.1 – Multivariable linear regression analyses for the relationship of categorized serum TCDD (ppt) with measures of physical functioning, Seveso Women's Health Study, Italy, 1996-2008. Cutpoints based on quartiles in full SWHS cohort.

	Physical Functioning									
	Time to flip 5 coins (sec) (n=152)		Walking speed (ft/sec) (n=148)		Reach down test time (sec) (n=151)		Grip strength dominant hand (kg) (n=98)		Grip strength non-dominant hand (kg) (n=98)	
TCDD exposure level (ppt, lipid-adjusted)	Adj.β[a]	95% CI	Adj.β[a]	95% CI	Adj.β[a]	95% CI	Adj.β[a]	95% CI	Adj.β[a]	95% CI
3.1 – 20.0	ref		ref		ref		ref		ref	
20.1 – 47.0	-1.44*	(-2.56, -0.32)	-0.00	(-0.23, 0.22)	-0.15	(-0.63, 0.34)	1.83	(-1.80, 5.46)	3.39	(-1.01, 7.78)
47.1 – 135.0	-0.56	(-1.97, 0.86)	-0.06	(-0.29, 0.17)	-0.27	(-0.76, 0.23)	3.58*	(-0.20, 7.37)	5.42*	(0.83, 10.02)
>135	-0.10	(-1.62, 1.43)	0.02	(-0.25, 0.28)	0.04	(-0.61, 0.69)	1.01	(-3.39, 5.40)	1.92	(-2.85, 6.69)
Test for trend	p=0.39		p=0.87		p=0.96		p=0.56		p=0.49	

[a] Adjusted for age at interview, primary wage earner education in 1996, BMI in 1996

* $p<0.05$

Supplementary Table 1.2 – Multivariable linear regression analyses for the relationship of serum TCDD (log10) with measures of working memory span, stratified by exposure before peak brain development (age 25), Seveso Women's Health Study, Italy, 2008–2009.

	All women (n=457)		**<25 years old in 1976 (n=299)**		**25+ years old in 1976 (n=158)**		
Measurement	Adj.β[a]	95% CI	Adj.β[a]	95% CI	Adj.β[a]	95% CI	P_{int}
Digit Span							
Forward	0.18	(-0.09, 0.45)	0.24	(-0.10, 0.57)	0.31	(-0.18, 0.81)	0.78
Backward	0.06	(-0.22, 0.34)	0.14	(-0.21, 0.49)	-0.03	(-0.18, 0.82)	0.60
Spatial Span							
Forward	0.05	(-0.21, 0.31)	0.22	(-0.10, 0.53)	-0.19	(-0.69, 0.31)	0.23
Backward	-0.03	(-0.30, 0.24)	0.11	(-0.23, 0.44)	-0.17	(-0.68, 0.34)	0.58

[a] Models adjusted for age at interview, primary wage earner education in 2008

Supplementary Table 1.3 – Multivariable linear regression analyses for the relationship of serum TCDD (log10) with measures of working memory span, stratified by menopause status in 2008, Seveso Women's Health Study, Italy, 2008–2009.

	All women (n=457)		**Premenopause (n=216)**		**Postmenopause (n=241)**		
Measurement	Adj.β[a]	95% CI	Adj.β[a]	95% CI	Adj.β[a]	95% CI	P_{int}
Digit Span							
Forward	0.18	(-0.09, 0.45)	0.22	(-0.19, 0.62)	0.28	(-0.13, 0.68)	0.45
Backward	0.06	(-0.22, 0.34)	0.03	(-0.41, 0.46)	0.12	(-0.29, 0.52)	0.57
Spatial Span							
Forward	0.05	(-0.21, 0.31)	0.27	(-0.10, 0.65)	-0.02	(-0.41, 0.36)	0.56
Backward	-0.03	(-0.30, 0.24)	0.07	(-0.38, 0.52)	0.01	(-0.37, 0.39)	0.58

[a] Models adjusted for age at interview, primary wage earner education in 2008

Supplementary Table 1.4 – Targeted Maximum Likelihood Regression (tmle) regression analyses for the relationship of quartiles of serum TCDD (ppt) with measures of working memory span, Seveso Women's Health Study, Italy, 2008–2009.

	Digit span				**Spatial Span**			
	Forward		Backward		Forward		Backward	
TCDD exposure level (ppt, lipid-adjusted)	Adj.β[a]	95% CI	Adj.β[a]	95% CI	Adj.β[a]	95% CI	Adj.β[a]	95% CI
3.1 – 20.0	ref		ref		ref		ref	
20.1 – 47.0	-0.06	(-0.85, 0.78)	-0.53	(-1.48, 0.28)	0.29	(-0.45, 1.05)	-0.22	(-1.13, 0.63)
47.1 – 135.0	0.42	(-0.33, 1.52)	-0.22	(-0.85, 0.78)	0.01	(-0.64, 0.78)	0.14	(-0.88, 1.24)
>135	0.54	(-0.21, 1.29)	-0.21	(-0.45, 1.05)	0.32	(-0.38, 1.21)	-0.23	(-1.19, 0.66)

[a]Models adjusted for age at interview, primary wage earner education in 2008

Superlearner library included SL.glm, SL.step, and SL.glm.interaction algorithms.

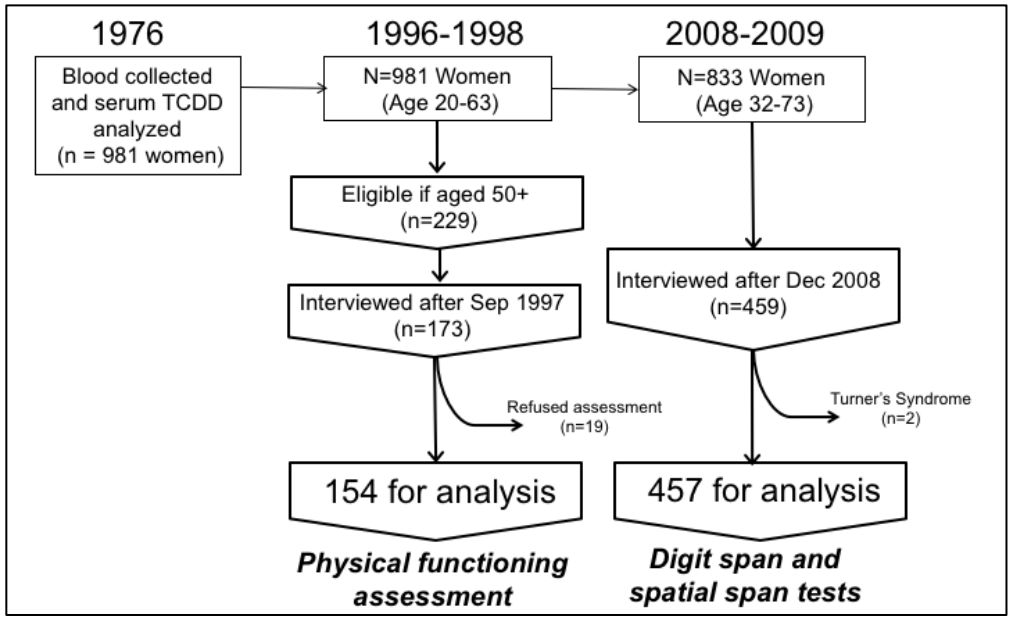
Supplementary Table 1.5– Multivariable linear regression analyses for the relationship of categorized serum TCDD (ppt) with measures of working memory, Seveso Women's Health Study, Italy, 1996-2008. Cutpoints based on quartiles in full SWHS cohort.

	Digit Span				**Spatial Span**			
	Forward		Backward		Forward		Backward	
TCDD exposure level (ppt, lipid-adjusted)	Adj.β[b]	95% CI	Adj.β[b]	95% CI	Adj.β[b]	95% CI	Adj.β[b]	95% CI
3.1 – 20.0	ref		ref		ref		ref	
20.1 – 47.0	-0.13	(-0.70, 0.44)	-0.39	(-0.94, 0.16)	0.13	(-0.35, 0.61)	-0.22	(-0.83, 0.39)
47.1 – 135.0	0.24	(-0.32, 0.80)	-0.17	(-0.71, 0.37)	0	(-0.49, 0.49)	0.05	(-0.55, 0.65)
>135	0.27	(-0.29, 0.82)	-0.19	(-0.74, 0.36)	0.15	(-0.35, 0.65)	-0.18	(-0.77, 0.40)
Test for trend		p_{trend}=0.12		p_{trend}=0.89		p_{trend}=0.73		p_{trend}=0.84

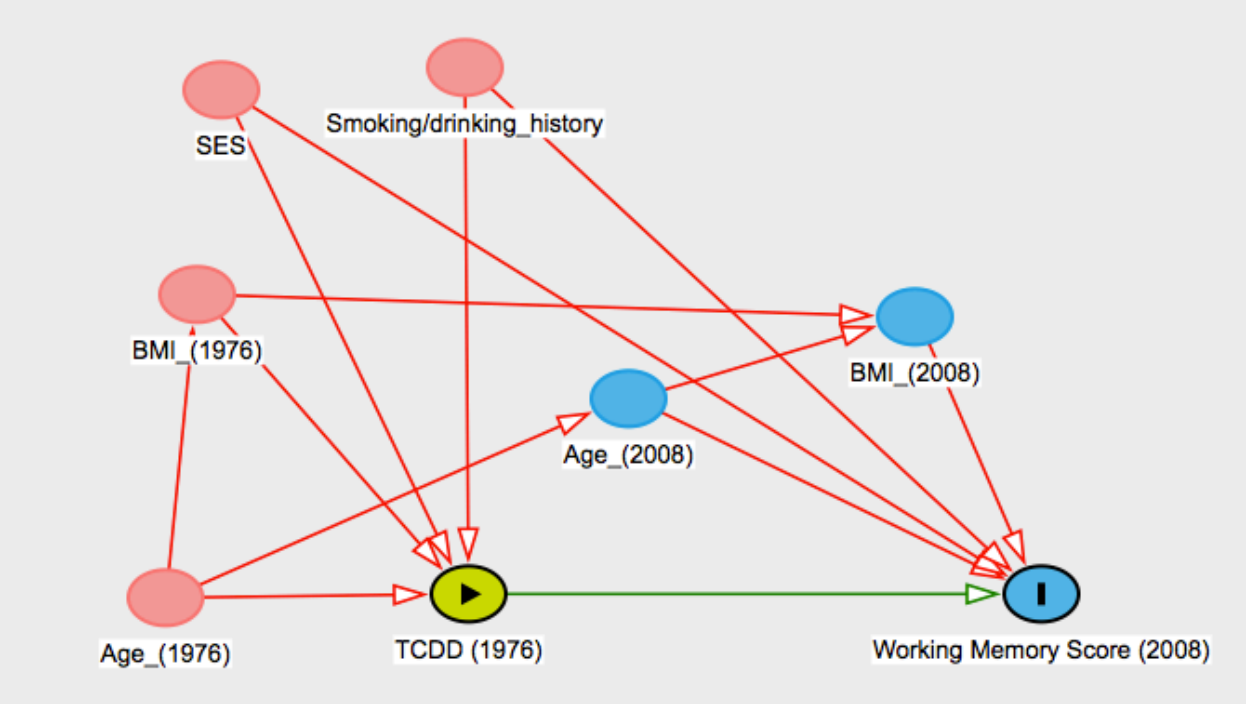
[b]Models adjusted for age at interview, primary wage earner education in 2008

1.10.2 Supplementary Figures

Supplementary Figure S1.1: Flowchart of sample selection for 1996 physical functioning assessment and 2008 working memory assessment in the SWHS.



Supplementary Figure S1.2: Example Directed acyclic graph (DAG). The endogenous variables in this system are denoted by the treatment (►, dioxin exposure), the outcome, I (i.e. score on memory subtest), and our a priori confounders, (pink). Neurocognitive and physical functioning in the SWHS:



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2. Prenatal Dioxin Exposure and Neuropsychological Functioning in the Seveso Second Generation Health Study

2.1 ABSTRACT

Background: Prenatal 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure has been shown to alter sexual differentiation of the brain in animal models, impacting pubertal development, behavior, cortical dominance, and cognition. The effects of early life exposure to dioxin-like compounds on human neurodevelopment, however, are less clear and warrant further investigation. Methods: The Seveso Women's Health Study (SWHS), initiated in 1996, is a well-characterized cohort of 981 Italian women who lived in proximity to an industrial accident in July 1976 that resulted in the highest residential TCDD exposures on record. In 2014-2016, we enrolled offspring born after the accident into the Seveso Second Generation Health Study. Children aged 7-17 years old (n=161) completed a neuropsychological assessment spanning executive function and reverse learning (Wisconsin Card Sort), non-verbal intelligence (Raven's Progressive Matrices), attention and hyperactivity (Connor's Continuous Performance), and memory (Rey's Auditory Verbal Learning). We used multivariate regression with robust standard error estimates accounting for clustering of siblings to model the associations between these outcomes and prenatal exposure defined as TCDD measured in maternal serum collected soon after the explosion and estimated to pregnancy. Results: The children (82 male, 79 female) averaged 13.1 (±2.9) years of age. Adjusting for confounders, a 10-fold increase in maternal serum TCDD was not adversely associated with reverse learning/set-shifting, memory, attention/impulsivity, or non-verbal intelligence. In sex-stratified models, prenatal TCDD was associated with more non-perseverative errors in boys but not in girls (p_{int}=0.04). TCDD was also associated with attention deficits on the CPT but only among children with the shortest breastfeeding histories. Conclusions: Possible differential neurotoxic sensitivities to TCDD by sex and lactation history may warrant confirmation in future studies.

2.2 INTRODUCTION

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a widespread environmental pollutant commonly produced as an unwanted by-product of industrial combustion processes [1]. TCDD is an endocrine disruptor that is highly lipophilic, chemically stable, and crosses the placenta [2,3]. Perinatal exposure has been linked to altered immune function, glucose regulation, and steroidogenesis, as well as dental developmental anomalies in humans and animals [4]. The diversity of health effects associated with TCDD is attributed to its high binding affinity for the aryl hydrocarbon receptor (AhR), a key transcription factor integral to many biological processes throughout human development, including cell growth, apoptosis, and detoxification [5].

TCDD's neurotoxicity is well documented in animals [6,7 8]. For example, studies in zebrafish have demonstrated TCDD's capacity to disrupt gene expression in highly conserved pathways of neuron development and brain growth [9-11]. Other experimental evidence links perinatal TCDD exposure to altered neurogenesis [12-14], as well as to behavioral and cognitive effects such as hyperactivity and impaired learning, memory, executive function, and motor development in rodents and monkeys [15-20]. Further, TCDD's action through the neuroendocrine pathways of AhR have been shown to alter sexual differentiation of the brain in animal models, impacting pubertal development, behavior, cortical dominance, and sex-specific changes in learning and memory [21-23].

Epidemiological studies have associated perinatal background exposures to mixtures of TCDD and related members of the dioxins family (e.g. polychlorinated dibenzodioxins (PCDDs) and dibenzofurans (PCDFs)) with subtle neurodevelopmental decrements in cognition, attention and language skills in offspring [24-28]. Additionally, background exposures to specifically dioxin-like congeners of polychlorinated biphenyls (PCBs) have been associated with delayed psychomotor development [29], hyperactivity, and poorer attention in children and adolescents [30-32], albeit inconsistently [33]. Higher exposures to dioxins, such as from the 1979 mass contamination of rice bran oil with PCBs and PCDFs in Yu-Cheng, Taiwan, have been linked to behavioral problems and lower cognition in perinatally exposed children compared to community-matched unexposed controls [34-36]. Autistic traits and poorer cognitive and motor development have also been reported in Vietnamese infants born in areas with residual dioxin contamination decades after the wartime use of Agent Orange [37-40]. These prior studies have assessed prenatal exposure to dioxins using proxies such as breastmilk levels [37-40], exposure registries [34,35], and estimates from dietary intake [26,27]; none have collected contemporaneous biomarkers near the time of the mother's highest exposure.

Despite a large body of research examining PCBs and dioxins on child neurodevelopment, the particular contribution of TCDD, the most toxic congener of this family of planar halogenated hydrocarbons, is not well studied. Furthermore, questions remain about the sex-specific effects of TCDD on human neurodevelopment since some studies have reported no sex differences or conflicting results as to which sex appears more sensitive[21,26,27,31,41]. In the present study, we examine the neuropsychological functioning of children whose mothers were exposed to TCDD as a result of an explosion in Seveso Italy on July 10, 1976 and who participated in the Seveso Women's Health Study [42,43]. We measured TCDD in blood collected near the time of exposure as well as estimated serum levels to the time of pregnancy. We also determine whether these potential neurotoxic effects are modified by child sex and breastfeeding histories since lactation could be an important source of postnatal exposure.

2.3 METHODS

Study Population

Recruitment of the Seveso Women's Health Study (SWHS) has been described previously [44]. Briefly, this historical cohort study recruited 981 women who were newborn to 40 years of age on July 10, 1976, resided at that time in the highest contaminated areas (Zones A and B) in Seveso, Italy, and had an adequate amount of stored serum collected soon after the explosion for analysis of TCDD. In 2014-2016, we enrolled 611 children of SWHS participants who were born after the explosion in the Seveso Second Generation Health Study (66.4% of 920 alive and eligible)[45]. Participants in the offspring study were 2-38 years old at enrollment, and completed a personal interview, a fasting blood draw, and anthropometry during their study visit. Participants who were 7-17 years old at the time of their enrollment were invited to participate in an assessment of neuropsychological functioning. This sample included 161 children (65% of 249 eligible) born to 120 mothers (See Supplementary Figure 2.1).

Procedures

The study was approved by the Institutional Review Boards of the participating institutions. We obtained, prior to participation, written informed consent from all mothers, oral assent from children aged 7-12 years, and written assent from children aged 13-17 years. Information on self-reported demographic, lifestyle factors and medical history were obtained

from a questionnaire administered to the mother in private by a trained nurse-interviewer. Meanwhile, a second trained interviewer conducted a brief medical exam with the child, which included anthropometric and blood pressure measurements, and administered the neuropsychological assessment in a quiet, private room. All interviewers were blinded to participants' maternal serum TCDD levels and zones of residence.

Neuropsychological Assessment

The neuropsychological assessment targeted domains hypothesized to be sensitive to dioxin exposure based on the findings from previous toxicological and human studies [12,15-20,30-33,36]. The main areas of interest included executive functioning and reversal learning, non-verbal intelligence, attention and hyperactivity, and memory. Every child was administered the neuropsychological assessment in the same order by one of two interviewers under identical conditions. The entire assessment included both computer and paper-based administration with a total duration of 50 minutes. The battery is described below with tests in order of administration.

Rey's Auditory Verbal Learning Test (RAVLT)

In this task, children listened to an audio recording of the test administrator reading a list of 15 semantically unrelated items. Immediately afterward, they were asked to recall as many of the words as they could. This process was repeated twice more and the child's immediate recall score was calculated as the sum of the number of words recalled across each of the three trials (i.e. scores could span 0-45). In the delayed recall task, conducted thirty minutes later, the children were asked to freely recall as many of the words as they could without hearing the list a fourth time (i.e. scores could span 0-15).

Ravens Progressive Matrices Test (RPM)

In the Ravens Progressive Matrices Test (RPM), children worked through a series of 60 visual problems requiring recognition of spatial, numerical, and stylistic patterns [46]. The multiple choice test, split into 5 sets of 12 puzzles of progressing difficulty, was administered with pen and paper. The RPM measures non-verbal intelligence, abstract reasoning, and problem-solving ability. Given the time constraints of the study visit, we implemented a 20-minute timed version of the exam. Those matrices the child left blank, perhaps due to the time limit, were counted as incorrect. Prior research indicates that this adaptation is a reasonable predictor of performance on the untimed version [47].

Connor's Continuous Performance Test II (CPT II)

The children completed the Connor's Continuous Performance Test (CPT II), a computerized task, that assesses impulse control, vigilance, and attention deficit hyperactivity disorder (ADHD)-like behaviors [48]. We examined continuous *t*-scores (standardized to a non-clinical population; mean=50 with a SD=10) for commission errors (false positives), omission errors (non-response, false negatives), and overall and between-set hit rate standard errors. Variability in hit rate, a measure of sustained attention on the test, reflects inconsistent performance, a symptom of ADHD [49]. We also examined the ADHD Confidence Index score, a continuous measure of the probability that the child would be diagnosed as having clinical ADHD.

Wisconsin Card Sorting Test (WCST)

Lastly, we administered a computerized version of the Wisconsin Card Sorting Test (WCST). This test evaluates set-shifting— a complex skill pertinent to executive function involving strategic planning, cognitive shifting/learning, and impulse control [46]. Subjects are tasked with sorting cards, one at a time, into one of four piles based on the shape, color, or

number of symbols on the card. However, the sorting rule is concealed and must be discovered by the subject through trial and error. As the test progresses, the current sorting rule changes without notice, requiring subjects to recognize and adapt their sorting accordingly. We examined raw scores for the number of trials taken to complete the first set and t-scores for total errors, non-perseverative errors, and perseverative errors [50].

TCDD Analysis

In utero TCDD exposure was defined in two ways: 1) the initial (1976) maternal TCDD level and 2) maternal serum TCDD extrapolated to the time of pregnancy. The former examines the hypothesis that the primary dose in 1976 resulted in a persistent and, if involving the epigenetics of her oocytes, possibly heritable change to the woman's reproductive system impacting the health of her offspring. The latter examines an alternative hypothesis that the toxicologically-relevant dose is the maternal body burden at the time of the pregnancy, which is influenced by initial dose, age, and other covariates [45,51].

Archived maternal serum samples collected in 1976 were stored at -20ºC until shipped to the Centers for Disease Control and Prevention (CDC) for analysis [44,52]. TCDD was measured by high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) methods [53] and adjusted for blood lipid concentrations prior to statistical analysis [54]. Serum TCDD levels were reported in picograms per gram lipid or parts per trillion (ppt). Samples below the limit of detection (average LOD = 18.8 ppt) were assigned a value equal to one-half of the LOD [55].

Maternal serum TCDD at pregnancy was estimated from a first order kinetic model based on TCDD concentrations in maternal serum collected in 1976, as well as from additional blood samples collected from a subsample of the mothers at the 1996 or 2008 follow up visits [45,51]. Serum TCDD in 1996 and 2008 were also measured with HRGC/HRMS methods with an average LOD of 1ppt [51]. The pregnancy TCDD estimate was extrapolated from the measurement collected closest to the pregnancy; as a result, estimates were extrapolated from 1976 blood samples for 15 children, from 1996 samples for 142 children, and from 2008 samples for 4 children. The median time between the closest TCDD measure and pregnancy was 5.5 years (IQR: 3.0–8.5 years), and 80% of the children had a maternal TCDD measurement within one TCDD half-life (9.0 years) of their births.

Statistical Analysis

Because the maternal serum TCDD distribution was approximately log-normal, the TCDD levels in 1976 and those extrapolated to the time of pregnancy were $\log_{10}$ transformed. The transformed serum TCDD levels were analyzed as a continuous exposure variable. For each endpoint, we assessed the shape of the dose-response function with locally weighted scatterplot smoothing (LOWESS) in Stata 13 (StataCorp, College Station, TX). We constructed multivariable linear regression models for each neuropsychological outcome and we fit the regression models with variances determined by the Huber-White sandwich estimator to account for clustering of siblings in the sample [56].

Covariates were identified *a priori* as confounders in the relationship between *in utero* TCDD exposure and neurocognitive performance with directed acyclic graphs (DAGs) informed by the literature (Supplementary Figures 2.2 and 2.3). Variables considered included the child's exact age at exam (in months), educational attainment of the primary wage earner in the child's home (<highschool, highschool, and > highschool) as a proxy for socioeconomic status, birth

order, the short form of the Home Observation for Measurement of the Environment [HOME (continuous measure combining maternal report and interviewer observations, standardized within our sample using z-scores)] [57], months breastfed, maternal smoking during pregnancy (yes/no), the mother's performance on an abbreviated 12-item Raven's Continuous Matrices test (continuous) [58,59], and maternal age at pregnancy. We examined whether there was any significant variability associated with either of the assessment's administrators or testing laptops. We also examined models adjusted for additional predictors of the outcome such as the child's video and computer game usage (maternal report of average hours per a week) and collected information on color-blindness as well as illness and medications used in the 24 hours preceding the assessment.

We evaluated child sex as a pre-specified effect modifier in both stratified regression analyses and with an interaction term with $\log_{10}$TCDD. We also considered modification by breastfeeding (0-1 month and ≥1 month), as lactation is possibly neuroprotective but also a significant source of postnatal exposure [60,61]. In sensitivity analyses, we also considered breastfeeding duration stratified at 0-3 month and ≥3 months, the median duration.
Main effects of TCDD were assessed for statistical significance at the 5% level and interactions were considered significant if the Wald test p-value for the cross-product term was <0.2.

2.4 RESULTS

Descriptive characteristics of the mother-child pairs included in this analysis are presented in Table 2.1. All children were Caucasian and the mean age at assessment was 13.1 (±2.9) years. Approximately half of the children were female (49%) and the majority (67.3%) were breastfed past the first month of infancy. As reported by the mother, 13 children had been diagnosed with a learning disability and 10 children with ADHD but none was on medication. The 120 SWHS mothers of the children in this sample were all relatively young at the time of the Seveso accident (mean=7.6 years old in July 1976 with an age range of newborn to 17 years old). Only 7% of the women reported smoking during pregnancy.

Distributions of maternal 1976 TCDD and levels estimated to the time of pregnancy are included in Supplementary Table 2.1. The median (IQR) of maternal 1976 and estimated pregnancy TCDD levels were 74.6 (40.4, 214.0) ppt and 4.5 (2.7, 9.2) ppt, respectively. Distributions of the neuropsychological outcomes are available in Supplementary Table 2.2. On the timed RPM, 55 children were unable to complete all 60 matrices within the time allotted. These children did not significantly differ in age or TCDD exposure from those who completed the test.

The lowess plots and models using restricted cubic splines, did not show evidence of non-linearity in the association of TCDD and neuropsychological performance so we proceeded with linear multivariate regression, with both outcome and exposure included as continuous variables. Tables 2.2 and 2.3 report adjusted beta coefficients and 95% CI for models regressing differences in test performance on maternal TCDD levels in 1976 serum and estimated pregnancy levels, respectively. A 10-fold increase in maternal 1976 serum TCDD was not significantly associated with performance on the WCST, CPT, RAVLT, or RPM (Table 2.2). We observed similarly null associations with all neuropsychological measures when we used maternal serum TCDD estimated to pregnancy (Table 2.3).

Sex differences

Significant heterogeneity by sex was observed between maternal 1976 TCDD and several measures of the WCST, namely total errors, trials to complete the first sorting set, and non-perseverative errors (Table 2.2). The coefficients on maternal 1976 TCDD indicated a dose-related pattern of poorer performance in boys but better performance in girls on these WCST measures (Tables 2.2); however, only the beneficial association between trials to complete first set in girls was significant (adj-β=-7.70, 95% CI -14.08, -1.33). With maternal TCDD estimated to pregnancy, the sex differences on the WCST were similar though none of the interactions were significant (Table 2.3).

Interaction by duration of breastfeeding

In models examining interaction and stratification by duration of breastfeeding (0-1 month versus ≥1 month), we observed no evidence that breastfeeding duration modifies the relationship between maternal 1976 TCDD and any of the neurodevelopmental measures (Table 2.4). However, there was evidence of interaction between breastfeeding duration and maternal TCDD estimated to the time of pregnancy for several scales of the CPT, including commission and omission errors, hit rate standard error overall, and the ADHD confidence index (Table 2.5). In stratified models, estimated pregnancy TCDD was significantly adversely associated with hit rate standard error overall (adj-β=-6.40, 95% CI 0.44, 12.37) and the ADHD confidence index (adj-β=10.91, 95% CI 0.22, 21.59) only among children with the shortest breastfeeding histories (Table 2.5). Coefficients in the group with longer breastfeeding, were null, indicating no adverse association with higher TCDD. Interactions of TCDD and breastfeeding with respect to the hit rate SE overall and ADHD confidence index persisted with some attenuation when the breastfeeding cutpoint was set to the median duration of ≥3 months.

2.5 DISCUSSION

This study examined the relationship of *in utero* exposure to TCDD and child neurodevelopment in a unique cohort of Italian children born to women exposed as a result of a 1976 explosion in Seveso, Italy. Although we previously found no association between postnatal TCDD exposure and working memory in the SWHS women [62], we hypothesized that those exposed *in utero*, a critical period of brain development, may be more sensitive to TCDD's neurotoxicity. Using serum TCDD levels measured in maternal samples collected soon after the explosion and near the time of the pregnancy, we observed no adverse relationships of maternal TCDD exposure with performance of their 7 to 17 year old children on several tasks of working verbal memory, attention, impulsivity, non-verbal intelligence, and reverse learning. While sensitivities among certain subgroups (e.g. children with shorter lactation histories and boys) merit closer attention, to date, there is limited evidence that TCDD is associated with adverse neuropsychological functioning in Seveso women and their children.

Although studies have found delays in cognitive and psychomotor skills observable at 6-12 months of age related to low levels of prenatal dioxin exposure [25,29,63,64], the associations in two of these earlier studies attenuated or disappeared by later infancy [63,64]. In somewhat older children 3 to 8 years of age, prenatal exposures to background levels of dioxin-like chemicals have been associated with no [26,65,66] to subtle but statistically significant deficits in language development, attention, and processing speed as well as atypical gender-related play behavior [27,31,67]. However, some of the most persistent cognitive impairments were reported in Yucheng, where 6-9 year old children were assessed following prenatal exposures to PCBs and furans in contaminated cooking oil [34,36]; while maternal exposures were not biologically measured in these

Taiwanese studies, previous work reports mean blood PCDFs levels in Yucheng patients of 0.76 ppb, approximately 10^5 times higher than background in Taiwan [68]. Taken together with our study, the literature suggests that neurodevelopmental decrements associated with *in utero* exposure may diminish as children grow older but the heterogeneity across these studies in populations, exposure profiles, and neuroassessment measures limits drawing firm conclusions.

Our study also found that child's sex may modify the relationship between TCDD and performance on the WCST with boys doing worse and girls doing better, although none of the adverse associations in boys were significant. Sex differences are plausible since TCDD's activation of AhR could result in endocrine disruption of the hypothalamic-pituitary-gonadal and thyroid axes during fetal brain development [69-71]. Furthermore, reports of sex-patterned alterations to neurogenesis [72-74] and neurobehavior in rodent models [15,20] suggest that perinatal dioxin exposure may play alternately anti-estrogenic and anti-androgenic roles in a sex-specific manner. Additional epidemiological evidence support that prenatal dioxins may have sexually dimorphic effects on human neurodevelopment [27,75,76]; while two of these studies corroborate greater attention and cognitive difficulties specifically among prenatally exposed boys [31,36], the literature also documents neurobehavioral effects specific to girls or opposing effects across sexes [27,75,76].

We also found that TCDD estimated at pregnancy was adversely associated with certain measures of attention (overall hit rate standard error and the ADHD confidence index on the CPT) only among children of women who breastfed for shorter time but not among children of those who breastfed longer. This finding appears consistent with previous work linking prenatal dioxins to poorer divided-attention [24] and ADHD-like inattention [31]. Although these studies did not examine effect modification by breastfeeding, their study participants reported, on average, shorter histories of breastfeeding than in our sample. Thus, if breastfeeding protects against the neurotoxic effects of TCDD, the relative difference in breastfeeding histories could explain why their studies observed associations at the marginal level while the present study did not.

Numerous studies have demonstrated the beneficial effects of breastfeeding on neurodevelopment [60,61]. Our concern was whether postnatal exposure via lactation to a lipophilic chemical could outweigh the benefits. For example, dioxin levels in breast milk, without accounting for duration of breastfeeding, were found to be associated with deficits in psychomotor and cognitive skills and increased autistic traits in young Vietnamese children [37-39]. However, our present findings, assuming no unmeasured confounding, suggest that the benefit of breastfeeding may negate the potential neurotoxic influence of perinatal dioxin exposure. This agrees with prior research on other endocrine-disrupting compounds (EDCs) indicating that prenatal exposures have a more pronounced deleterious influence on neurodevelopment in childhood than lactational exposures incorporating breastfeeding duration [24,30,67,77].

Our study has some limitations. Though our findings, particularly with respect to sex and lactation differences, are suggestive, the small sample size, made effectively smaller in stratified analyses and when taking sibling covariance into account, limits conclusive inference. Therefore, we cannot rule out the possibility of sampling error in explaining the significant findings. The number of outcomes examined also raises the possibility of observing type I error from multiple comparisons. While we did not correct for multiple comparisons, we did observe patterns across related psychometric domains and redundancies of the assessment that were qualitatively consistent. Likewise, analyses may have been underpowered given the wide age range. For example, while the adverse direction of the coefficients between maternal estimated pregnancy TCDD levels and correct responses on the RPM were consistent with other studies of this

relationship in Yucheng (ages 6-9, n=118) and Mohawk children (ages 10-16, n=271), our study was relatively smaller within these age groups with wider confidence intervals spanning the null [36,78].

The reliance on estimated levels of dioxin during pregnancy is likely a source of exposure misclassification but we expect bias to be non-differential with respect to the outcome. Another limitation is that although our neuroassessment contained a diverse cross-section of psychometric domains with hypothesized sensitivity to TCDD based on the literature [12,15-20,30-33], we possibly still omitted the most sensitive endpoints of cognition and behavior. For example, we could not consider play behavior, psychomotor skills, and language development— outcomes reported in previous epidemiological studies of prenatal dioxin exposure— due to the older ages of our sample [27,64,75,76].

We found little evidence of selection bias despite the relatively low participation among eligible 7-17 year old children in the 2014 SWHS follow up (65%). Among non-participant children (n=88 children, 68 mothers), maternal 1976 levels were higher than those among participants (median(IQR)=157.5(50.8, 393.5)ppt vs. 74.6 (40.4, 214.0) ppt) but estimated exposures in pregnancy were not significantly different (median(IQR)=4.9 (3.0, 9.1)ppt vs. 4.5 (2.7, 9.2) ppt). Non-participant children also tended to be younger and to have mothers who were younger at the time of the accident, explaining the higher 1976 levels [79]. Furthermore, participation was unlikely to be related to the outcome as the prevalence of maternally reported ADHD and learning disability in the analytic sample (7% and 10%, respectively) was only slightly higher than rough estimates of ADHD prevalence previously reported in Italian children (5%) [80]. Nevertheless, if the mothers' 1976 levels are more relevant, exclusion of these children with higher maternal 1976 levels could have led us to underestimate the relationship between the mother's highest level of exposure and neurodevelopment in the Seveso second generation..

Due to study constraints, we were unable to administer the neuropsychological assessment to SWHS children aged 18+, many of whom were born closer to the time of their mother's initial exposure. Thus, it is possible that the findings in our sample of 7-17 year old children, who were born several TCDD half-lives after the accident, are not generalizable to these older children who may have experienced the highest *in utero* exposures. Given that some outcomes were related to pregnancy TCDD levels in the present study, a neuropsychological examination of these older children may be warranted.

This study also has several strengths. The unique exposure scenario of the Seveso population, which resulted in a wide distribution of TCDD exposures independent of other dioxin-like compounds [81], allows isolation of the particular neurotoxic properties of TCDD. Further, given the substantial decline in environmental dioxins since 1976, the possibility of high postnatal exposures beyond lactation in this study population is low. These conditions allowed us to consider maternal highest lifetime dose of TCDD and levels estimated at the time of pregnancy as two potentially distinct biological pathways impacting the fetus. While we observed suggestive differences in the associations between 1976 and estimated pregnancy exposure models of TCDD with the children's neuropsychological outcomes, the pattern was not consistent and is difficult to interpret without more research.

2.6 CONCLUSIONS

The Seveso second generation cohort is unique in its wide range of biologically measured TCDD exposures and affords one of the only epidemiological opportunities to evaluate the specific effects of TCDD exposure *in utero* on neuropsychological function. While we found limited evidence of an adverse association between prenatal dioxin exposure and

neuropsychological functioning in 7-17 year old children, the neurotoxicological profile of TCDD warrants further research. In particular, our findings of susceptibility differences by sex and lactation histories, particularly with respect to psychometric measures of learning and attention, should be explored in future work. Additional research that includes the adult children in the second generation, many of whom received the highest prenatal exposures, may also better reveal the long-term neuropsychological effects of prenatal TCDD exposure across the life-course.

2.7 ACKNOWLEDGEMENTS

This study was supported by grants F06 TW02075-01 from the National Institutes of Health, R01 ES07171 and 2P30-ESO01896-17 from the National Institute of Environmental Health Sciences, R82471 from the U.S. Environmental Protection Agency, and 2896 from Regione Lombardia and Fondazione Lombardia Ambiente, Milan, Italy.
We acknowledge the significant contributions in sample analyses made by D. Patterson, L. Needham, and W. Turner to the Seveso Women's Health Study. We gratefully acknowledge Hospital of Desio field staff for data collection. We also thank Dr. Susan Schantz and our colleagues at Helix for their early expert advice in developing the neuroassessment panel and the X-Lab at UC Berkeley for loaning laptops used to administer the neuroassessment.

2.8 TABLES

Tables 2.1-2.5 Prenatal Dioxin Exposure and Neuropsychological Functioning in the Seveso Second Generation Health Study

Table 2.1: Characteristics of mothers and children in the study sample, Seveso Second Generation Health Study, Seveso, Italy, 2014-2016

Characteristic	N	%
Maternal characteristics	120	(100)
Age at explosion		
0-10	80	(66.7)
11-20	40	(33.3)
Maternal age at delivery		
24-29	29	(18.01)
30-34	76	(47.2)
35-43	56	(34.78)
Total parity		
0	35	(21.7)
≥1	126	(78.3)
Maternal education		
<Highschool	28	(23.3)
Highschool	34	(28.3)
>Highschool	58	(48.3)
Smoking during pregnancy		
Smoker	11	(6.8)
Non-smoker	150	(93.2)
Household characteristics		
Primary wage earner education		
<Highschool	37	(30.8)
Highschool	43	(35.8)
>Highschool	40	33.4
Children characteristics	161	(100)
Child sex		
Female	79	(49.1)
Male	81	(51.9)
Child age at assessment		
7-10	29	(18)
11-13	49	(30.4)
14-17	83	(51.6)
Low birthweight (<2500g)	17	(10.6)
Preterm (<37wks)	14	(8.7)

Table 2.1: (Continued)

Breastfeeding duration (months)		
0-1 month	52	(32.7)
> 1 month	109	(67.3)
Sibling groups in neuro sample		
One child groups	83	(51.6)
Two sibling groups	33	(41)
Three sibling groups	4	(7.4)
Diagnosed with ADHD (maternal report)	11	(6.9)
Diagnosed with learning disability (maternal report)	15	(9.4)

Table 2.2: Neuropsychological outcomes associated with a 10-fold increase in maternal serum TCDD levels in 1976 (Log_{10}TCDD) for all children, stratified by child sex, Seveso Second Generation Health Study, Seveso Italy 2014-2016

Outcome	Direction of poorer performance[c]	All children n=161 adj-β[a] 95% CI	Girls n=79 adj-β[a] (95% CI)	Boys n=82 adj-β[a] (95% CI)	p_{int}
Wisconsin Card Sorting Test (WCST)					
Total errors T-score	(-)	1.32 (-1.81, 4.45)	4.78 (-0.46, 10.01)	-1.84 (-5.85, 2.16)	0.01
Trials to complete first set	(+)	-1.71 (-6.33, 2.90)	-7.70 (-14.08, -1.33)*	2.70 (-3.90, 9.30)	0.01
Perseverative errors T-score	(-)	0.53 (-3.37, 4.43)	1.72 (-5.00, 8.45)	-0.29 (-5.78, 5.20)	0.29
Non-perseverative errors T-score	(-)	1.22 (-2.06, 4.49)	3.56 (-2.02, 9.14)	-1.46 (-5.49, 2.56)	0.04
Connor's Continuous Performance Test (CPT)[b]					
Commission errors T-score	(+)	-0.69 (-3.62, 2.24)	-1.99 (-6.11, 2.12)	0.56 (-3.87, 4.99)	0.22
Omission errors T-score	(+)	-1.08 (-4.37, 2.21)	-1.54 (-7.03, 3.94)	-0.05 (-3.50, 3.40)	0.34
Hit rate SE overall T-score	(+)	0.47 (-2.44, 3.39)	0.35 (-4.41, 5.12)	1.32 (-2.24, 4.88)	0.41
Hit rate SE between sets T-score	(+)	-0.15 (-3.09, 2.80)	-0.33 (-5.26, 4.59)	0.57 (-3.00, 4.14)	0.61
ADHD confidence index	(+)	-0.20 (-5.27, 4.87)	1.32 (-7.23, 9.87)	0.09 (-5.72, 5.91)	0.75
Rey's Auditory and Verbal Learning Test (RAVLT)					
Immediate recall	(-)	1.08 (-0.50, 2.66)	1.04 (-1.49, 3.58)	1.01 (-0.98, 3.00)	0.76
Delayed recall	(-)	0.32 (-0.34, 0.98)	0.28 (-0.70, 1.27)	0.30 (-0.64, 1.24)	0.93
Ravens Progressive Matrices (RPM)					
Total correct	(-)	0.10 (-2.03, 2.24)	0.28 (-3.45, 4.01)	-0.11 (-2.71, 2.49)	0.97

[a] Adjusted for child age at assessment, maternal age at pregnancy, highest level of education in household, maternal shortened Ravens score, total parity, child sex

[b] Excluding child with apparent non-effort or computer malfunction during assessment

[c] (+) higher scores indicate poorer performance on test, (-) lower scores indicate poorer performance on test

* $p<0.05$

Table 2.3: Neuropsychological outcomes associated with a 10-fold increase in maternal serum TCDD levels estimated at the time of pregnancy (Log_{10}TCDD), stratified by child sex, Seveso Second Generation Health Study, Seveso Italy 2014-2016

Outcome	Direction of poorer performance[c]	All children n=161 adj-β[a] 95% CI	Girls n=79 adj-β[a] (95% CI)	Boys n=82 adj-β[a] (95% CI)	p_{int}
Wisconsin Card Sorting Test (WCST)					
Total errors T-score	(-)	-0.13 (-3.52, 3.26)	1.02 (-4.19, 6.22)	-2.51 (-6.95, 1.92)	0.56
Trials to complete first set	(+)	0.56 (-3.77, 4.89)	-1.91 (-7.60, 3.78)	3.00 (-3.71, 9.71)	0.40
Perseverative errors T-score	(-)	0.95 (-3.70, 5.59)	1.91 (-5.30, 9.12)	-1.67 (-8.25, 4.91)	0.60
Non-perseverative errors T-score	(-)	-0.48 (-3.75, 2.80)	0.04 (-4.24, 4.33)	-2.04 (-6.49, 2.42)	0.60
Connor's Continuous Performance Test (CPT)[b]					
Commission errors T-score	(+)	-1.52 (-4.80, 1.76)	-1.97 (-6.80, 2.86)	-0.66 (-5.58, 4.25)	0.82
Omission errors T-score	(+)	0.06 (-2.47, 2.58)	0.61 (-3.49, 4.72)	0.87 (-1.94, 3.68)	0.95
Hit rate SE overall T-score	(+)	2.47 (-0.49, 5.43)	3.26 (-1.72, 8.25)	2.87 (-0.41, 6.15)	0.84
Hit rate SE between sets T-score	(+)	1.07 (-2.27, 4.42)	0.84 (-4.31, 5.99)	2.47 (-2.03, 6.97)	0.63
ADHD confidence index	(+)	3.92 (-1.17, 9.01)	6.60 (-1.93, 15.12)	3.50 (-1.94, 8.94)	0.49
Rey's Auditory and Verbal Learning Test (RAVLT)					
Immediate recall	(-)	1.02 (-0.65, 2.68)	0.10 (-2.56, 2.75)	1.66 (-0.55, 3.87)	0.58
Delayed recall	(-)	0.46 (-0.19, 1.12)	0.02 (-0.93, 0.96)	0.81 (-0.18, 1.79)	0.49
Ravens Progressive Matrices (RPM)					
Total correct	(-)	-1.95 (-4.02, 0.11)	-2.86 (-5.81, 0.10)	-2.02 (-5.23, 1.20)	0.50

[a] Adjusted for child age at assessment, maternal age at pregnancy, highest level of education in household, maternal shortened Ravens

[b] Excluding child with apparent non-effort or computer malfunction during assessment

[c] (+) higher scores indicate poorer performance on test, (-) lower scores indicate poorer performance on test

* $p<0.05$

Table 2.4: Neuropsychological outcomes associated with a 10-fold increase in maternal serum TCDD levels in 1976 (Log_{10}TCDD), stratified by breastfeeding duration (0-1 month vs. >1 month), Seveso Second Generation Health Study, Seveso Italy 2014-2016

		All children	**0-1 Months**	**>1 Month**	
		n=161	n=52	n=109	
Outcome	**Direction of poorer performance[c]**	**adj-β[a] 95% CI**	**adj-β[a] (95% CI)**	**adj-β[a] (95% CI)**	**p_{int}**
Wisconsin Card Sorting Test (WCST)					
Total errors T-score	(-)	1.32 (-1.81, 4.45)	0.41 (-6.75, 7.57)	2.06 (-1.69, 5.81)	0.94
Trials to complete first set	(+)	-1.71 (-6.33, 2.90)	2.73 (-8.47, 13.93)	-3.57 (-8.86, 1.72)	0.66
Perseverative errors T-score	(-)	0.53 (-3.37, 4.43)	0.86 (-9.50, 11.23)	1.59 (-2.79, 5.96)	0.86
Non-perseverative errors T-score	(-)	1.22 (-2.06, 4.49)	1.43 (-5.23, 8.09)	0.46 (-3.41, 4.34)	0.26
Connor's Continuous Performance Test (CPT)[b]					
Commission errors T-score	(+)	-0.69 (-3.62, 2.24)	0.94 (-4.86, 6.73)	-0.63 (-4.18, 2.91)	0.60
Omission errors T-score	(+)	-1.08 (-4.37, 2.21)	1.08 (-3.65, 5.81)	-2.02 (-6.53, 2.49)	0.59
Hit rate SE overall T-score	(+)	0.47 (-2.44, 3.39)	2.93 (-2.31, 8.18)	-0.36 (-4.04, 3.33)	0.31
Hit rate SE between sets T-score	(+)	-0.15 (-3.09, 2.80)	1.31 (-3.43, 6.06)	-1.03 (-4.88, 2.81)	0.21
ADHD confidence index	(+)	-0.20 (-5.27, 4.87)	4.85 (-5.13, 14.83)	-2.61 (-8.99, 3.77)	0.20
Rey's Auditory and Verbal Learning Test (RAVLT)					
Immediate recall	(-)	1.08 (-0.50, 2.66)	-0.21 (-2.73, 2.31)	1.71 (-0.19, 3.61)	0.51
Delayed recall	(-)	0.32 (-0.34, 0.98)	0.05 (-1.02, 1.12)	0.46 (-0.36, 1.29)	0.69
Ravens Progressive Matrices (RPM)					
Total correct	(-)	0.10 (-2.03, 2.24)	0.52 (-3.11, 4.15)	0.29 (-2.44, 3.03)	0.73

[a] Adjusted for child age at assessment, maternal age at pregnancy, highest level of education in household, maternal shortened

[b] Excluding child with apparent non-effort or computer malfunction during assessment

[c] (+) higher scores indicate poorer performance on test, (-) lower scores indicate poorer performance on test

* $p<0.05$

Table 2.5: Neuropsychological outcomes associated with a 10-fold increase in maternal serum TCDD levels estimated at the time of pregnancy (Log_{10}TCDD), stratified by breastfeeding duration (0-1 month vs. >1 month), Seveso Second Generation Health Study, Seveso Italy 2014-2016

Outcome	Direction of poorer performance[c]	All children n=161 adj-β[a] 95% CI	0-1 Month n=52 adj-β[a] (95% CI)	>1 Month n=109 adj-β[a] (95% CI)	p_{int}
Wisconsin Card Sorting Test (WCST)					
Total errors T-score	(-)	-0.13 (-3.52, 3.26)	-0.75 (-8.17, 6.66)	0.35 (-3.52, 4.23)	0.93
Trials to complete first set	(+)	0.56 (-3.77, 4.89)	-1.41 (-9.77, 6.95)	1.05 (-3.72, 5.82)	0.91
Perseverative errors T-score	(-)	0.95 (-3.70, 5.59)	-0.79 (-10.97, 9.39)	3.12 (-2.73, 8.97)	0.56
Non-perseverative errors T-score	(-)	-0.48 (-3.75, 2.80)	0.59 (-4.49, 5.66)	-1.91 (-6.20, 2.38)	0.37
Connor's Continuous Performance Test (CPT)[b]					
Commission errors T-score	(+)	-1.52 (-4.80, 1.76)	1.41 (-4.13, 6.94)	-3.28 (-7.40, 0.83)	0.12
Omission errors T-score	(+)	0.06 (-2.47, 2.58)	2.42 (-2.71, 7.55)	-1.98 (-5.17, 1.22)	0.10
Hit rate SE overall T-score	(+)	2.47 (-0.49, 5.43)	6.40 (0.44, 12.37)*	-0.22 (-3.99, 3.55)	0.05
Hit rate SE between sets T-score	(+)	1.07 (-2.27, 4.42)	2.20 (-3.44, 7.84)	-0.11 (-4.43, 4.20)	0.53
ADHD confidence index	(+)	3.92 (-1.17, 9.01)	10.91 (0.22, 21.59)*	-1.92 (-7.74, 3.89)	0.02
Rey's Auditory and Verbal Learning Test (RAVLT)					
Immediate recall	(-)	1.02 (-0.65, 2.68)	0.18 (-2.23, 2.59)	1.67 (-0.80, 4.14)	0.32
Delayed recall	(-)	0.46 (-0.19, 1.12)	0.50 (-0.28, 1.28)	0.44 (-0.59, 1.47)	0.94
Ravens Progressive Matrices (RPM)					
Total correct	(-)	-1.95 (-4.02, 0.11)	-3.42 (-7.15, 0.31)	-0.61 (-3.56, 2.34)	0.30

[a] Adjusted for child age at assessment, maternal age at pregnancy, highest level of education in household, maternal shortened Ravens score, total parity, child sex

[b] Excluding child with apparent non-effort or computer malfunction during assessment

[c] (+) higher scores indicate poorer performance on test, (-) lower scores indicate poorer performance on test

* $p<0.05$

2.9 FIGURES

No figures

2.10 SUPPLEMENTARY MATERIALS

2.10.1 Supplementary Tables

S.Table 2.1-2.2: Prenatal Dioxin Exposure and Neuropsychological Functioning in the Seveso Second Generation Health Study

Supplementary Table 2.1: Distributions of maternal TCDD concentrations (ppt, lipid-adjusted) in serum collected near 1976 and estimated to the time of the pregnancy, Seveso Second Generation Health Study, 2014-2016

	n	Geometric mean	95%CI	min	25th	50th	75th	max
All children								
1976 maternal concentration	161	99.0	(161.5, 127.7)	5.5	40.4	74.6	214.0	9140.0
Estimated concentrations in pregnancy	161	4.6	(3.9, 5.5)	0.3	2.7	4.5	9.0	620.5
Male								
1976 maternal concentration	82	94.3	(64.1, 138.6)	5.5	40.6	73.9	211.0	9140.0
Estimated concentrations in pregnancy	82	3.8	(3.0, 4.8)	0.3	2.0	3.9	7.1	130.5
Female								
1976 maternal concentration	79	103.6	(73.4, 146.3)	9.9	35.0	75.2	244.0	2820.0
Estimated concentrations in pregnancy	79	5.7	(4.4, 7.4)	0.3	2.9	5.6	10.1	620.5

Supplementary Table 2.2: Distributions of neuropsychological test scores for all children and stratified by child sex, Seveso Second Generation Health Study, 2014-2016

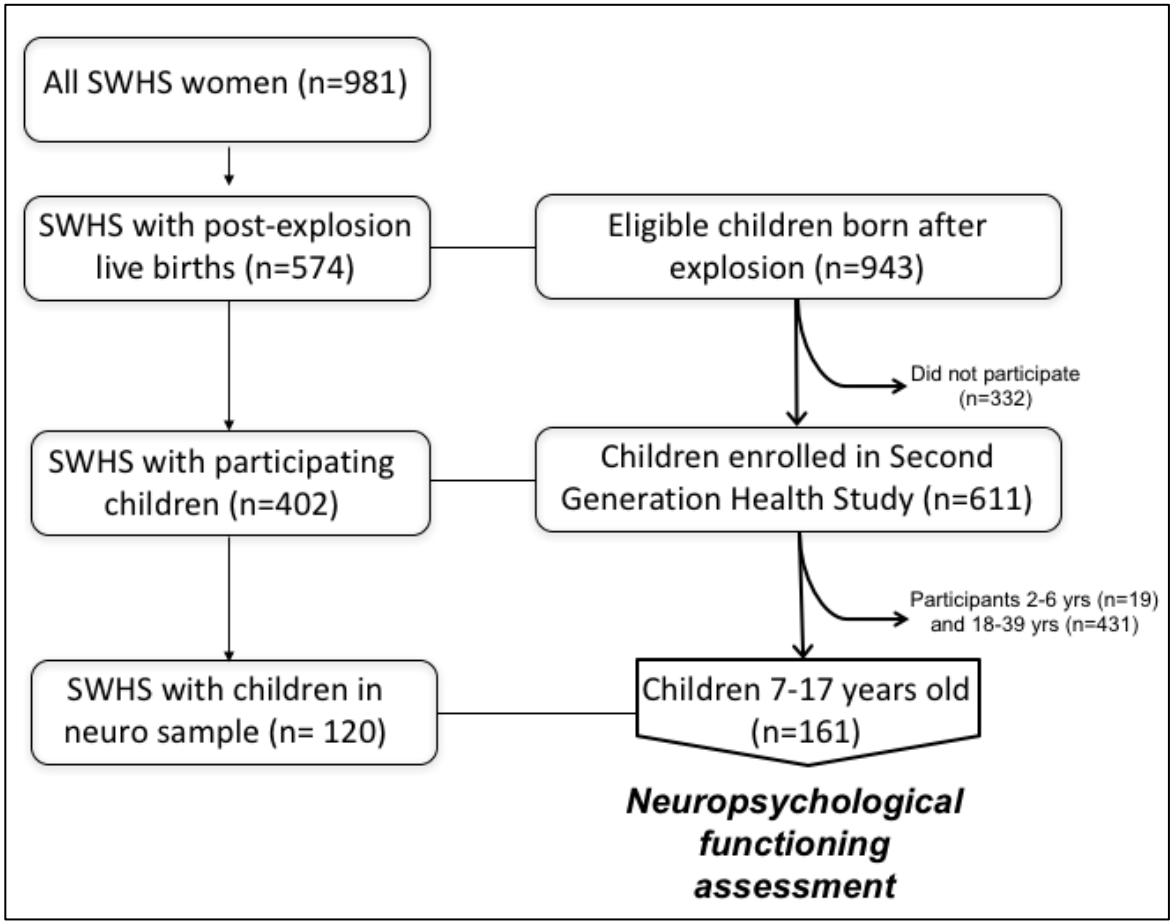
Outcome	All children (n=161) Mean (±sd)	Male (n=82) Mean '(±sd)	Female (n=79) Mean '(±sd)
Wisconsin Card Sorting Test (WCST)			
Total errors T-score	49.6 (12.1)	48.1 (12.3)	51.2 (11.8)
Trials to complete first set	18.7 (15.6)	19.6 (16.6)	17.7 (14.5)
Perseverative errors T-score	52.1 (16.2)	50.5 (16.7)	53.7 (15.7)
Non-perseverative errors T-score	50.5 (11.8)	49.7 (12.3)	51.3 (11.3)
Connor's Continuous Performance Test (CPT)[a]			
Commission errors T-score	48.8 (11.4)	47.0 (11.8)	50.7 (10.8)
Omission errors T-score	47.4 (7.6)	47.9 (8.)	46.9 (7.2)
Hit rate SE overall T-score	47.2 (9.9)	48.4 (10.4)	45.9 (9.3)
Hit rate SE between sets T-score	48.6 (9.8)	49.3 (10.)	47.9 (9.6)
ADHD confidence index	44.6 (17.2)	51.8 (16.1)	37.0 (15.1)
Rey's Auditory and Verbal Learning Test (RAVLT)			
Immediate recall	27.2 (5.5)	27.1 (5.3)	27.3 (5.7)
Delayed recall	10.2 (2.5)	10.0 (2.6)	10.4 (2.3)
Ravens Progressive Matrices (RPM)			
Total correct	41.7 (9.3)	41.6 (9.4)	41.7 (9.2)

[a] Excluding one child with apparent non-effort or computer malfunction during assessment

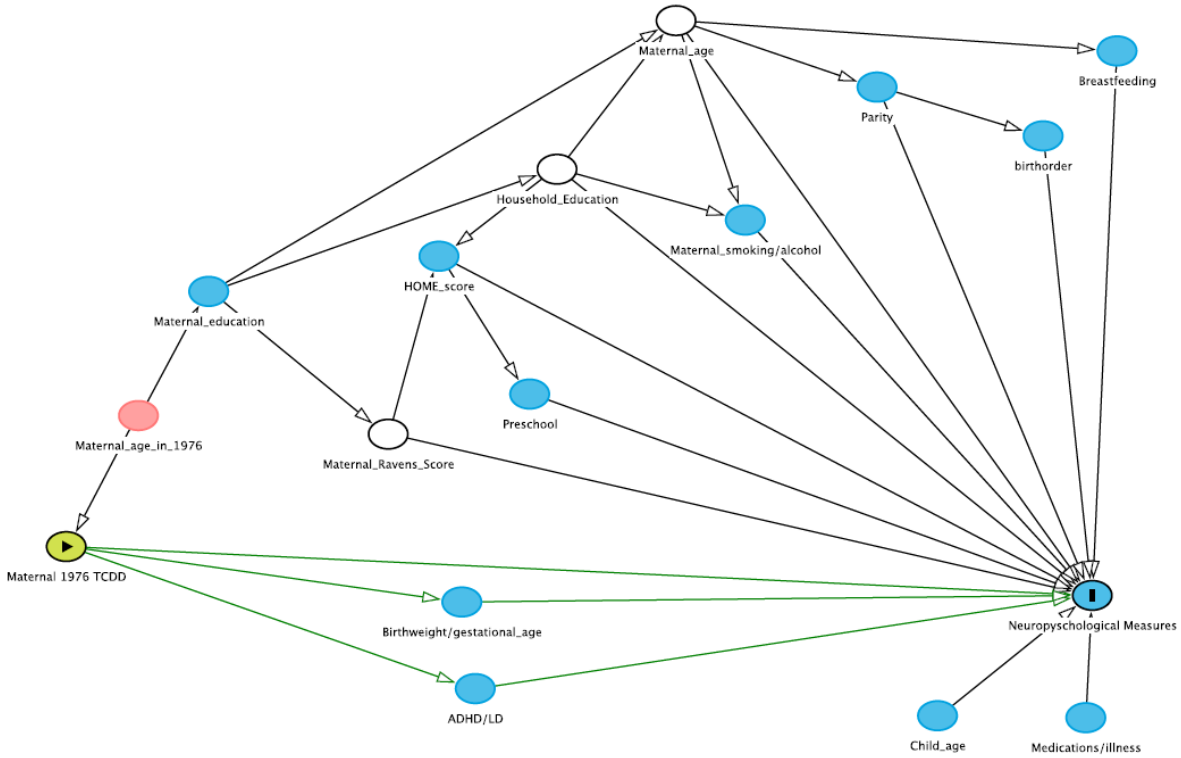
2.10.1 Supplementary Figures

S.Figure 2.1-2.3: Prenatal Dioxin Exposure and Neuropsychological Functioning in the Seveso Second Generation Health Study

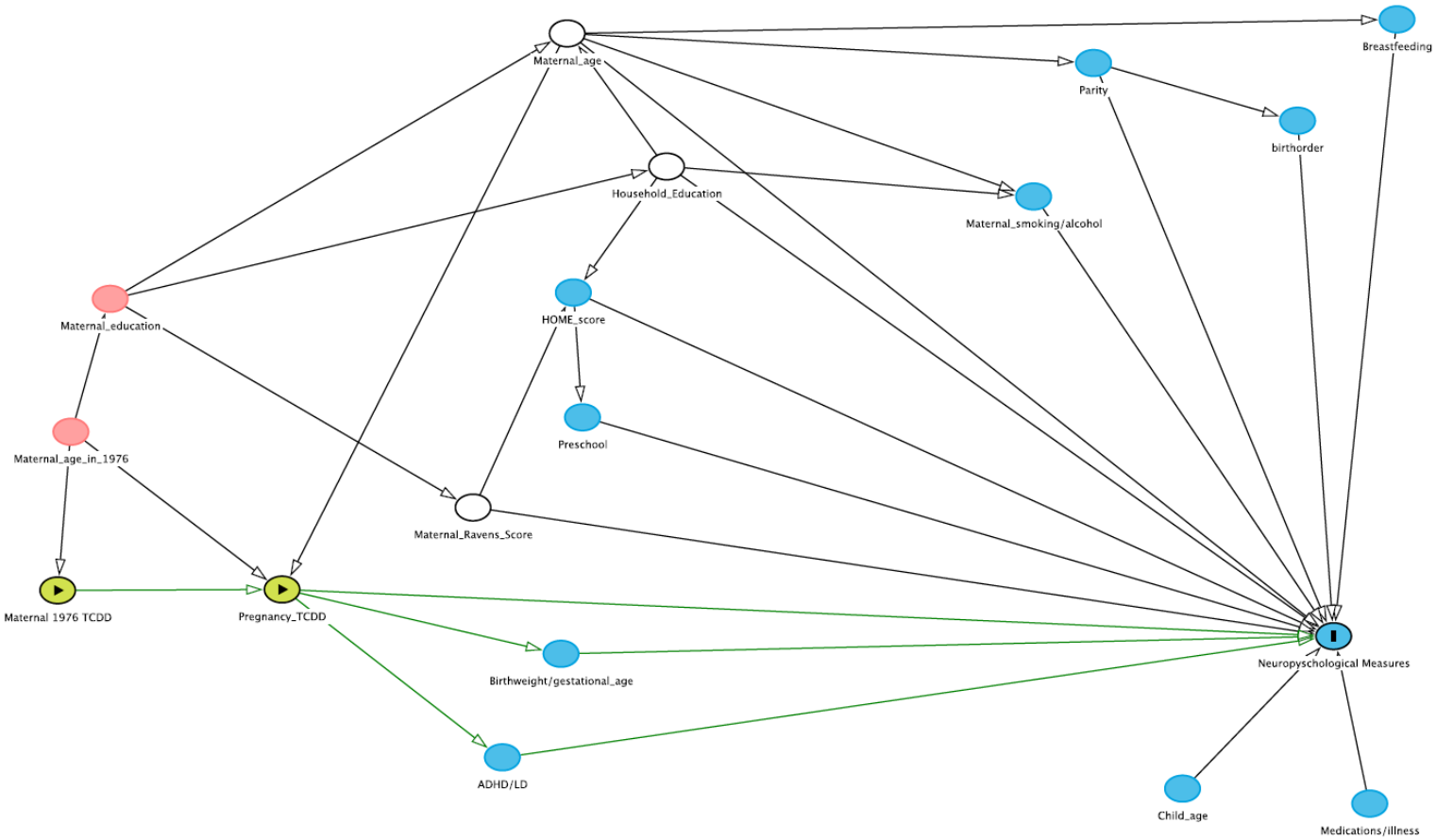
Supplementary Figure S2.1: Flowchart of study sample participating in neuropsychological assessment in Seveso Second Generation Study.



Supplementary Figure S2.2: Example directed acyclic graph (DAG) of the association between maternal 1976 TCDD levels and neuropsychological functioning in children. The endogenous variables in this system are denoted by the treatment (▶, dioxin exposure), the outcome, I (i.e. performance on neuropsychological test), our a priori confounders, (pink and white), and adjustment set (white). Exogenous, unmeasured variables in the system that may potentially confound the relationship of interest are omitted:



Supplementary Figure S2.3: Example directed acyclic graph (DAG) of the association between maternal TCDD levels estimated to pregnancy and neuropsychological functioning in children. The endogenous variables in this system are denoted by the treatment (►, dioxin exposure), the outcome, I (i.e. performance on neuropsychological test), our a priori confounders, (pink and white), and adjustment set (white). Exogenous, unmeasured variables in the system that may potentially confound the relationship of interest are omitted:



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3. *AHR* Gene-Dioxin Interactions and Birthweight in the Seveso Second Generation Health Study

3.1 ABSTRACT

Background: 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is proposed to interfere with fetal growth via altered activity of the aryl hydrocarbon receptor (protein: AHR, gene: *AHR*) pathway which regulates diverse biological and developmental processes including xenobiotic metabolism. Genetic variation in *AHR* is an important driver of susceptibility to low birthweight in children exposed to prenatal smoking, but less is known about these genetic interactions with TCDD, AHR's most potent xenobiotic ligand.
Methods: The Seveso Women's Health Study (SWHS), initiated in 1996, is a cohort of 981 Italian women exposed to TCDD from an industrial explosion in July 1976. We measured TCDD concentrations in maternal serum collected close to the time of the accident. In 2008 and 2014, we followed up the SWHS cohort and collected data on birth outcomes of SWHS women with post-accident pregnancies. We genotyped 19 single nucleotide polymorphisms (SNPs) in *AHR* among the 574 SWHS mothers.
Results: Neither SNPs nor TCDD exposure alone were significantly associated with birthweight. However, we found 6 individual SNPs in *AHR* that modified the association between maternal TCDD and birthweight, implicating gene-environment interaction. We saw an even stronger interaction when we examined the joint contribution of these SNPs in a risk allele score. These SNPs were all located in noncoding regions of *AHR*, particularly in proximity to the promoter.
Conclusions: This is the first study to examine how genetic variation across the *AHR* gene may shape fetal susceptibilities to dioxin exposure.

3.2 INTRODUCTION

In 1976, an industrial accident near Seveso Italy resulted in one of the highest residential exposures to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in history [1-5]. TCDD, a common by-product of industrial and combustive processes, is a persistent organic pollutant, and a known carcinogen [6] and endocrine disruptor [7]. TCDD has been shown to cross the placenta [8,9] and *in utero* exposure to TCDD and dioxin-like chemicals has been linked in animal studies to altered immune function, glucose regulation, steroidogenesis, and neurobehavioral and bone development [10-18].

Animal studies have also suggested that TCDD may impact fetal growth [14,19-22], possibly via altered activity of the aryl hydrocarbon receptor (protein: AHR, gene: *AHR*), a nuclear receptor and transcription factor active in many tissues including the placenta [23-28]. Upon binding TCDD in the cell cytoplasm, AHR moves to the nucleus, where it induces expression of several xenobiotic metabolizing enzymes, such as cytochrome P450, and exhibits cross-talk with pathways of hormone synthesis [29]. In addition to detoxification, the AHR pathway regulates myriad biological processes related to development, cell growth, apoptosis, and immune function [30].

Of the epidemiologic studies that have examined biological markers of maternal dioxin exposures and birthweight, three found no association [31-33], another three found adverse associations [34-36], and three had adverse associations that did not reach statistical significance [37-39]. In addition, we previously reported a suggestive yet not statistically significant inverse association (adjusted-$\beta = -47.7$, 95%CI: −107.3, 11.9 for a 10-fold increase in TCDD) [37,40]

between the post-1976 serum dioxin concentrations of women living in Seveso, Italy at the time of the accident, and the birthweight of their first births after the accident. The lack of consistency across the literature examining the effects of TCDD on birthweight may be due to the wide variation in sample size, difference in exposure levels, and perhaps, genetic variation represented in these study populations.

Inter-strain and interspecies differences in AHR ligand binding affinities suggest that genetic variation in *AHR* may influence susceptibility to TCDD [41]. Human evidence supporting this hypothesis is drawn from two Japanese studies that found that a polymorphism in maternal *AHR* (rs2066853) conferred significant reductions in birthweight in pregnant women who smoked cigarettes (components of cigarette smoke, such as benzo(a)pyrene, also bind AHR) [42]. A more recent study of Japanese infants (n=421) that examined the relationship between this SNP and prenatal dioxins toxic equivalency (TEQ) reported no relationship with birthweight [43] but consideration of additional *AHR* SNPs in larger cohorts with higher exposures to specifically TCDD, the most potent compound of the TEQ, is warranted.

In the present analysis, we investigate whether maternal *AHR* gene variation modifies the association between maternal exposure levels of TCDD and birthweight in the children born after the Seveso explosion to mothers who participated in the Seveso Women's Health Study (SWHS), a follow-up study of women living in Seveso, Italy at the time of the accident.

3.3 METHODS

Study Population and Procedures

In 1996, 20 years after the explosion, the Seveso Women's Health Study was initiated. Eligible women were aged 40 years or younger on July 10, 1976, resided in the most contaminated areas, and had blood samples collected soon after the explosion. A total of 981women (80% of eligible) participated [5]. In 2008 and 2014, we followed up these participants. Details of the study procedure for the 2008 and 2014 studies are described elsewhere [44,45]

At each follow-up visit, women were interviewed in a private room at the Hospital of Desio by a nurse interviewer who was blinded to participant TCDD levels. Information was obtained on medical and reproductive history with detailed information on each pregnancy and on demographic and lifestyle factors. Between 1976 and 2016, a total of 574 SWHS mothers reported 943 live-birth children born after the 1976 accident. We obtained genetic information on 567 mothers (98.4%), corresponding with 929 births. Seven women (who had 14 live births) either did not consent to biobanking their blood specimens or did not have adequate amounts of blood specimen for DNA isolation. We excluded an additional 27 multiple births and one singleton with missing birthweight, leaving 901 singletons from 562 mothers for the primary analyses (Table 3.1).The study was approved by the Institutional Review Boards of the participating institutions and we obtained written informed consent from all mothers prior to participation.

Outcome assessment

Birthweights and gestational duration were based on maternal report. In a small sample (n=139), we confirmed reported birthweights using hospital records. These data indicated that women slightly over-reported birthweight by 22g on average, but this was non-differential by TCDD exposure [40].

TCDD Analysis

TCDD was measured in archived maternal serum samples collected near the time of the explosion by high-resolution gas chromatography/high-resolution mass spectrometry methods at the Centers for Disease Control and Prevention (CDC) [46,47]. Details of the serum sample selection and TCDD concentrations are presented elsewhere [48,49]. Prior to statistical analysis, maternal serum TCDD levels were adjusted for blood lipid concentrations by dividing TCDD on a whole-weight basis by total serum lipid content, estimated from measurements of triglycerides and total cholesterol [50]. Serum TCDD levels were reported in picograms per gram lipid or parts per trillion (ppt). The median lipid-adjusted limit of detection (LOD) for the full population was 18.8 ppt. Samples below the LOD (9.4% in full cohort) were assigned a value equal to one-half of the LOD [51]. By considering the 1976 TCDD levels, we examine the hypothesis that the mother's primary dose permanently altered the reproductive system or oocytes of exposed women, possibly resulting in persistent epigenetic changes that could impact fetal growth [52].

SNP Selection and Genotyping

We used the HapMap browser [53] in the Caucasian population of European descent (CEPH) and the 1000 Genomes Toscani in Italia population (TSI) to choose SNPs from *AHR* that were expected to have minor allele frequencies greater than 5% in our Italian Caucasian sample. In cases where SNPs were in linkage disequilibrium, we sought an appropriate tagging SNP representative of this group of co-varying SNPs to conserve study power and resources. These candidate SNPs were further pared down to those with known or suspected functional relevance as reported in the literature or as listed in the open-access Regulome SNP database (Stanford University)[54]. We particularly prioritized SNPs linked to xenobiotic exposure and fetal development though we considered *AHR* SNPs associated with any health outcome. Our final genotyping assay comprised 18 SNPs across 50kb of the *AHR* gene as well as 1 SNP in *AHR*'s upstream intergenic region. Location of the SNPs and their physical distribution are presented in Table 3.2 and Supplemental Figure 3.1.

Maternal DNA for genotyping was isolated from archived blood using a QIAamp Blood DNA Maxi kit (QIAGEN, Valencia, CA, USA) with some modification as previously described [55]. High throughput genotyping of selected SNPs was performed using the multiplex platform iPLEX (Sequenom, San Diego, CA) at the Genomics Core at the University of Minnesota. The main steps involved multiplex PCR, single-base primer extension, and finally mass spectrometry to determine the genotype. Quality assurance procedures for genotyping included assessment of randomly distributed blank samples and duplicates of participant samples. Call rates were above 98% for all 19 SNPs. Samples with lower success rate were resolved with additional genotyping. All genotype distributions were in accordance with Hardy-Weinberg equilibrium assumptions.

Statistical Analyses

TCDD measures were analyzed as a log10-transformed continuous variable. We considered those covariates that were used in previous reports of birthweight in this cohort or identified *a priori* as confounders between *in utero* dioxin and birthweight in a directed acyclic graph (DAG). These covariates included maternal age at pregnancy (continuous, years), year of pregnancy, smoking during pregnancy (yes/no), parity (0, 1 or >2 pregnancies), maternal height (cm), pre-accident history of delivering a low birthweight infant, and child sex and gestational age (maternal report in weeks). Model parameters were estimated with use of generalized estimating equations (GEE) with exchangeable correlation of the variance structure to account

for siblings. We first re-evaluated the association between TCDD and birthweight in this analytical sample for comparison with our previously reported result of an adverse but non-significant relationship [37,40].

Prior to fitting our genetic models, linkage disequilibrium between SNPs was assessed with r^2 and observed SNP distributions and correlations were compared to those in the TSI of the 1000 Genomes. Genotype analyses considered two penetrance models with reasonable power given our sample sizes: a) additive allelic inheritance (i.e., groups inheriting 0, 1, or 2 minor alleles at each SNP) and b) dominant model (i.e., inheriting 0 vs. at least 1 minor allele). In models assuming dominant penetrance of the minor "variant" allele, the genotype at each SNP was analyzed as an indicator variable. In separate analyses assuming additive allelic penetrance, the genotype was analyzed categorically and if a dose-response pattern was observed with the increasing number of variant alleles (0, 1, 2), an ordinal variable was used. In both scenarios, the reference group was the genotype with 0 variant alleles.

To evaluate the main effects of each SNP, we fit multivariate models of birthweight regressed on maternal genotype controlling for the above covariates and TCDD levels. We then considered crude and adjusted models of interaction between maternal genotype and TCDD on birthweight by constructing a cross-product term of genotype and TCDD (i.e., $SNP_{Maternal}$ x TCDD). TCDD was considered as a continuous variable (log10-transformed) and as a categorized variable to explore GxE interaction allowing for nonlinear associations between TCDD and birthweight. The lowest TCDD category with 0 variant alleles served as the reference group. We examined cumulative associations across SNPs through calculation of a genetic risk allele score by summing, for each individual, the number of risk alleles (0 or 1 if ≥1 risk allele present) across all significant SNPs ($p_{int}<0.1$) in the individual SNP models and, in a sensitivity analysis, by weighting the SNP components by the size of their coefficients in the individual models. Alleles associated with lowered birthweight in interaction with TCDD were designated as the "risk" allele and summed across the genotypes at all SNPs included in the score. The score was then examined in models with and without interaction with maternal TCDD levels. The score, a common method that conserves study power and potentially gives insight on underlying biological mechanisms, was examined as a continuous variable [56].

Multiple births were excluded from the initial models, but were added back in a sensitivity analysis. Additional sensitivity analyses considered models excluding preterm (<37 weeks gestation) births and models restricted to the mother's first birth after the explosion using robust regression. All analyses were performed in Stata 13 (StataCorp LP. College Station, TX) with the exception of Hardy-Weinberg and linkage disequilibrium statistics and the Benjamini-Hochberg False Discovery Rate correction for multiple comparisons which were performed using the *SNP assoc* and *p.adjust* packages in R, respectively [57,58].

3.4 RESULTS

Descriptive statistics of study sample

Maternal 1976 serum TCDD concentrations across demographic and pregnancy characteristics are presented in Table 3.1. Mothers in this sample averaged 14.7 (±7.7) years of age at the time of the accident. The average maternal age at pregnancy across all 901 births was 29.6 years (±5.3) and 20% of women had a history of pregnancy prior to the accident. The majority of mothers (81.4%) had educational attainment beyond the compulsory education in Italy. Women who were younger in 1976 tended to have higher levels of serum TCDD, higher levels of educational attainment at follow-up, and were slightly older at time of pregnancy than

SWHS women who were adults at the time of the accident, a pattern described in previous studies of the SWHS [59,60].The median TCDD levels in maternal serum was 61.3 ppt (IQR: 29.0 163.0) in 1976. The mothers in our analytic sample (n=562) did not differ in sociodemographic characteristics or medical history from the sample of all mothers with eligible children in the Seveso Second Generation Health Study (n=574).

Among the 901 singleton births, the average birthweight was 3,264 (+/- 526) grams. The number of low birthweight and preterm infants was 57 (6.3%) and 62 (6.9%), respectively. Without considering genotype, a 10-fold increase in 1976 serum TCDD was associated with a non-significant reduction in birthweight (adjusted β=-33.37, 95% CI: -84.63, 17.88; p=0.20) adjusting for covariates. These findings are similar to what has been observed in previous studies in this cohort although marginally different due to the slightly different sample who had DNA available for analysis [37,40,45]

Minor allele frequencies for the 19 SNPs, shown in Table 3.2, ranged from 8.1% (rs17779352) to 41.2% (rs6968865). The allelic frequencies in the SWHS population were similar to those observed in the 1000 Genomes TSI [61]. Linkage disequilibrium was relatively low among the *AHR* SNPs, with 5% of pairwise LD comparisons exceeding an $r^2>0.8$ (see Supplementary Figure 3.1 for LD Plot).

Associations between SNPs and birthweight

We observed no association between any of the 19 SNPs in *AHR* on birthweight (Supplementary Table 3.1). The only SNP with a suggestive association was rs2066853. Under an additive model of penetrance, each additional risk allele (A) was associated with a -62 gram reduction in birthweight relative to the wildtype GG mothers ($p_{trend}= 0.08$). However, this trend was based on 7 mothers with the AA genotype and therefore a dominant model (adjusted β=-67.55, 95% CI: -145.46, 10.36; p=0.09) was used in subsequent sensitivity analyses to conserve power (Figure 3.1). The only other SNP (rs2237297) that was notably associated with birthweight (adjusted β=-58.55, 95% CI: -138.21, 21.10; p=0.15) is in high LD with rs2066853 ($r^2=0.93$) in this population.

Gene-environment interactions

In crude models, 4 of the 19 SNPs (rs6968865, rs3757824, rs10249788, rs2040623) exhibited interaction with 1976 maternal TCDD levels (Supplementary Table 3.2). When these models were adjusted for covariates, effect sizes were attenuated but these four SNPs and two others (rs2282885, rs2106728) suggested gene-environment interaction (Table 3.3). Further, these six interactive SNPs were clustered around the promoter and first and last introns of *AHR*. We observed 5 SNPs (rs6968865, rs3757824, rs10249788, rs4236290, and rs2040623) for which presence of the variant allele in mothers was associated with strong adverse associations between TCDD and birthweight relative to the reference group of homozygous wildtype mothers. For example, a 10-fold increase in TCDD was associated with 136.17g (95%CI: -244.72, -27.62; p=0.01) and 1.94g (95%CI: -60.02, 56.13; p=0.95) reductions in birthweight among children of mothers with the variant (CC/CT) and wildtype (TT) rs10249788 genotypes, respectively. This interactive association was particularly notable at the low and high ends of TCDD exposure where, continuing with the example of rs10249788, the variant allele was protective at low TCDD levels but crossed to adverse at higher TCDD exposures (Figure 3.2). We observed this pattern of GxE interaction with TCDD for three other SNPs (rs6968865, rs3757824, and

rs2040623). For two SNPs, rs2282885 and rs2106728, the variant allele exhibited a protective influence on the association between TCDD and birthweight.

We developed a genetic risk allele score to explore the joint association of multiple *AHR* SNPs in interaction with TCDD on birthweight. We considered the 6 SNPs that were interactive with TCDD for the risk allele score. Two of these SNPs (rs2040623 and rs2106728) located at the end of the *AHR* gene were excluded from the score as they were in high LD with two other SNPs already in the score, rs2282885 and rs3757824 (r^2>0.84), respectively, and did not contribute appreciably to the association or variance of the models. Thus, the final risk allele score was based on the 4 SNPs located in the intergenic and promoter regions only (rs6968865, rs3757824, rs10249788, rs2282885). Considered independently of dioxin interaction, the risk allele score had no association with birthweight (adjusted β=-5.00, 95% CI: -28.37, 18.38; p=0.68). However, the risk allele score modified the association of TCDD on birthweight (p_{int}= 0.002) (Figure 3.3). For example, a 10-fold increase in TCDD was not associated with birthweight among 199 children whose mothers had the lowest risk allele score (adjusted β=-2.51, 95% CI: -119.62, 114.60; p=0.97). In contrast, TCDD was associated with significant decreases in birthweight in children of mothers with scores greater than 1, with the largest reductions observed when mothers carried all 4 variants (adjusted β=-189.11, 95% CI: -315.70, -62.52; p=0.003).

In sensitivity analyses, excluding preterm births did not appreciably change the findings of interaction between the *AHR* gene and TCDD exposure (Supplementary Table 3.3) nor did including multiple births (Supplementary Table 3.4). When we restricted to the first post-explosion birth, the GxE coefficients were also comparable but with diminished statistical precision due to the smaller sample sizes (Supplementary Table 3.5).When we accounted for multiple testing with FDR adjustment, the significance of the interactions for individual SNPs was attenuated (Table 3.3); however, the GxE interaction by the risk allele score remained statistically significant.

3.5 DISCUSSION

In our previous research [37,40], we found that birthweight was suggestively related to maternal TCDD blood concentrations and hypothesized that there may be a susceptible subgroup that is at higher risk. In this study, we examined whether variation in maternal *AHR* genotypes, coding for a transcription factor with diverse functions including dioxin metabolism, could explain heterogeneity in the effects of *in utero* dioxin exposure on birthweight. We found interactions between maternal serum TCDD levels and 6 SNPs in *AHR*'s regulatory regions, particularly in proximity to the gene's promoter. We observed an even stronger interaction when we examined the joint contribution of these SNPs by a risk allele score. These novel results demonstrate for the first time an interactive association between *AHR* genetics and TCDD exposure on birthweight and add to the previous literature that variation in maternal *AHR* in interaction with smoking may influence fetal growth [42,62,63]. While the only extant report of maternal AHR genetics (specifically, a single SNP, rs2066853) on fetal susceptibility to dioxins suggested no interaction [43], our study, the first report in a population of European ancestry, builds upon this work by examining additional SNPs across the AHR gene and interaction across a wider distribution of TCDD in a population with background exposures to other dioxin-like compounds. Average reductions in birthweight associated with TCDD in our study were in some cases as large as those reported among maternal smokers within high risk genotypes (60-140g) in

previous studies. Susceptibility culminated in an average 189 g reduction in children of mothers carrying all four of the highest risk genotypes.

This finding is noteworthy given that the independent associations of neither TCDD nor maternal *AHR* genotypes with birthweight were significant. Specifically, we did not observe associations between the 19 *AHR* SNPs and birthweight, though the variant allele at rs2066853 was associated with a large but statistically non-significant weight reduction. This lack of a relationship is consistent with what is known from genome-wide association studies (GWAS) of birthweight; while AHR activity is integral to fetal development, no variants have been linked to altered fetal growth in GWAS [64,65]. Nevertheless, previous GWAS studies of birthweight have only considered child's genetics and not maternal genetics as examined here. This affirms the methodological caution advanced by Humblet et al that only testing for interaction among marginally significant SNPs may screen out and miss potentially important interactive loci in epidemiological studies [66].

The SNPs in the *AHR* risk allele score in our study may be important for development and toxicant metabolism. Previous experimental and epidemiologic research indicate that the 3 SNPs in proximity to *AHR*'s promoter region may influence expression of the *AHR* gene [67,68], suggesting a possible biological mechanism for the interaction we observed. For example, rs6968865, upstream of *AHR*, is a documented expression quantitative trait locus (eQTL) associated with expression of *AHR* in pancreas tissue (Genotype-Tissue Expression GTex Project, Broad Insitute). The minor allele (T) of rs10249788, a SNP in *AHR*'s promoter, has been associated with higher expression of *AHR* than the C allele in human blood [68] and downstream genes such as interleukin factors may be more upregulated in the presence of the TT genotype, independently of dioxin exposure [69]. A possible biological explanation for this expression difference is offered by a study in human endometrial cells which observed that nuclear factor 1-C (NF1C), a suppressor of *AHR* expression, preferentially bound to promoters with the C-allele compared to the T-allele at rs10249788 [70].To the best of our knowledge, no studies on *AHR* regulation by rs3757824, another significant promoter SNP in our study that is located about 2kb away from rs10249788, have been published, but the heterozygous genotype of this locus has been linked to higher risk of cryptorchidism in Italian boys [71], suggesting a role in fetal development.

The most widely studied *AHR* variant in humans is rs2066853, an Arg554Lys missense mutation in exon 10, the codon site for *AHR*'s transactivation domain (TAD). Insight into the possible biological mechanism of rs2066853 is offered by a study that observed significantly higher mRNA levels of *AHR* in human lymphocytes with the wildtype genotype compared to homozygotes Lys554Lys (AA) [72]. However, functional studies of the polymorphism linking variation at rs2066853 to altered *CYP1A1* or *AHR* expression are mostly inconclusive [73,74]. Though this SNP had the largest main effect on birthweight in our study, the association was borderline significant and we did not observe interaction of this SNP with dioxin exposure. This finding is consistent with a recent study in a Japanese cohort, which also did not report evidence of interaction between this SNP and dioxins nor TEQ on birthweight [43]. However, in this same Japanese cohort, rs2066853was associated with circulating levels of dioxins in pregnant mothers, suggesting that rs2066853 may still be related to efficiency of dioxin metabolism [75].

To date, there is limited evidence on the functional relevance of rs2106728, found in the gene's last exon, on *AHR* activity but one study reported a large but insignificant association of this SNP with endometriosis in Japanese women [76]. This SNP and others could be worthy of

further investigation in the SWHS where dioxin exposure was observed to be non-significantly associated with a doubling of endometriosis risk [77].

There are several possible pathways by which maternal xenobiotic-metabolizing genes such as *AHR* can alter the intrauterine environment and affect birthweight; for example, their activity contributes to circulating xenobiotic levels in maternal-fetal circulation, placental function, endocrine regulation, and/or indirectly through the fetus's detoxification capacity inherited from the mother's alleles [78]. The relative contributions of these pathways are difficult to disentangle but our study corroborates previous reports that maternal variation in other detoxifying proteins, particularly in the promoter region, may influence fetal susceptibility to toxicants [79,80]. For example, we previously found that maternal and child promoter variation in PON1, another multifunctional, detoxifying gene, has been linked to subsequent expression and detoxifying activity of organophosphate pesticides [81] and possibly modifies the relationship of prenatal organophosphate exposure and neurodevelopment [82,83]. However, whether *AHR* exhibits analogous genetic-epigenetic regulation at the site of the promoter and the contribution of the child's genetics is not yet known.

This study has several notable strengths including the long follow up of the SWHS through reproductive years and one of the largest sample sizes among analyses examining GxE with *AHR*. The wide exposure distribution of TCDD in our study population also allowed for higher resolution of significant gene-dioxin interactions, particularly at the low and high ends of exposure where the interaction was most prominent. In addition, a candidate gene approach focusing on the *AHR* gene, where the biological plausibility is strong, conserved study power by limiting the number of multiple tests. Lastly, the genetic homogeneity in the SWHS limits confounding by population stratification.

Our study has several limitations. First, this is the earliest report examining this GxE association in a Caucasian population but it should also be confirmed in additional cohorts. Second, though its potency for dioxin metabolism has been widely studied, AHR is just one player in a complex network of genes and proteins that mediate dioxin toxicity. Further, while we only considered 19 main *AHR* SNPs among approximately 150 SNPs with >1% MAF across human populations, we prioritized those evidencing functional significance for fetal development or detoxification. Third, in the interest of conserving study power, we used a dominant model of inheritance for all the risk alleles and an additive count of risk alleles across SNPs in composing the risk allele score on birthweight, assumptions which may not accurately reflect underlying biology. Future studies will investigate gene-dioxin interactions with respect to additional outcomes in the Seveso Women's Health Study and in the Seveso Second Generation and the roles of other genes in the AHR pathway as well as the child's genotype.

3.6 CONCLUSIONS

This is one of the first studies to evaluate gene-dioxin interactions in a large homogenous cohort with a broad range of exposure levels. We used a prospective study design of the SWHS, high quality TCDD measurements collected close to the time of the exposure, and an assessment of multiple polymorphisms across the *AHR* gene. We found six maternal *AHR* SNPs to significantly modify the relationship of maternal TCDD concentrations on child birthweight. *AHR* may explain variation in human sensitivity to dioxin exposure, particularly among the offspring of TCDD exposed mothers. Replication in additional cohorts or confirmation in mechanistic studies is warranted.

3.7 ACKNOWLEDGEMENTS

This work was supported by funding from the National Institutes of Health [grant number F06 TW02075-01]; National Institute of Environmental Health Sciences [grant numbers R01ES007171, R01ES007171-17S1, 2P30-ESO01896-17, and R24 ES028529]; U.S. Environmental Protection Agency [grant number R82471]; and Regione Lombardia and Fondazione Lombardia Ambiente Milan, Italy [grant number 2896]. Ms. Ames was supported by the Ruth L. Kirschstein Predoctoral Individual National Research Service Award (NIEHS) F31 Fellowship [grant number F31ES026488].

We acknowledge the significant contributions in sample analyses made by D. Patterson, L. Needham, and W. Turner to the Seveso Women's Health Study. We gratefully acknowledge Nicole Gelpi and Giulia Del Giorno for coordinating data collection at Hospital of Desio and Maria Escundero-Fung, Toshnia Hossain, and Michael Zhou for assistance in DNA isolations at the Children's Environmental Health Laboratory (Berkeley Global Campus, University of California, Berkeley). We also acknowledge the significant contributions to the iPlex assay design by Shalane Porter and her colleagues at the Genomics Core of the University of Minnesota.

3.8 TABLES

Tables 3.1-3.5: AHR Gene-Dioxin Interactions and Birthweight in the Seveso Second Generation Health Study

(see next page)

Table 3.1: Descriptive statistics of mothers with genetic data in the SWHS, 1996-2014

Characteristic	N	(%)	1976 Serum TCDD (ppt) Median (IQR)
Total women	562	(100.0)	61.3 (29.0, 163.0)
Total live births	901	(100.0)	
Age at explosion (years)			
0-10	164	(29.2)	157.5 (51.8, 320.5)
11-20	239	(42.5)	53.4 (25.4, 105.0)
21-30	139	(24.7)	41.3 (22.0, 80.9)
31-40	20	(3.6)	39.0 (27.7, 66.8)
Menarche status at explosion			
Premenarche	211	(37.5)	131.0 (50.5, 286.0)
Postmenarche	351	(62.5)	44.4 (22.5, 86.7)
Pre-explosion parity			
0	451	(80.3)	71.1 (31.7, 192.0)
1	71	(12.6)	36.6 (21.1, 70.4)
≥2	40	(7.1)	35.6 (24.5, 69.1)
Maternal education at last follow-up			
<Required	105	(18.7)	42.5 (23.5, 73.4)
Required/high school	432	(76.9)	64.7 (30.0, 188.0)
University	25	(4.5)	75.7 (30.2, 180.0)
Age at pregnancy (years)			
<25	152	(16.9)	46.4 (21.5, 103.5)
25-29	309	(34.3)	55.5 (28.6, 131.0)
30-34	270	(30.0)	67.2 (29.9, 187.0)
≥35	170	(18.9)	64.3 (35.0, 176.0)
Smoking during pregnancy			
No	813	(90.2)	61.2 (29.3, 164.0)
Yes	88	(9.8)	50.0 (20.7, 102.0)
Weight gain during pregnancy (kg)[a]			
<10	190	(21.1)	67.9 (38.2, 141.0)
10-14	421	(46.7)	54.3 (26.7, 136.0)
15-19	171	(19.0)	62.8 (22.0, 214.0)
≥20	96	(10.7)	70.9 (30.0, 204.0)
Low birthweight (<2500g)			
No	844	(93.7)	60.4 (28.4, 157.0)
Yes	57	(6.3)	64.7 (31.7, 131)
Preterm (<37 weeks)			
No	839	(93.1)	60.4 (28.4, 156.0)
Yes	62	(6.9)	76.2 (33.2, 206.0)
Infant sex			
Male	473	(52.5)	55.0 (27.2, 130.0)
Female	428	(47.5)	67.0 (29.9, 179.5)

[a]Missing data on pregnancy weight gain for 23 live births.

Table 3.2: Description of *AHR* SNPs genotyped in SWHS mothers, 1996-2014

SNP	Location (chr:bp)	Alleles	MAF (%)	Functional consequence
rs6968865	7:17247645	T/A	41.2	Upstream, intergenic region
rs3757824	7:17296411	A/G	18.5	intron, regulatory region variant
rs10249788	7:17298523	C/T	12.4	intron, regulatory region variant
rs713150	7:17300533	C/G	25.7	intron, regulatory region variant
rs17722841	7:17303970	G/C	14.2	intron
rs2282885	7:17305990	T/C	35.3	intron
rs3802083	7:17309283	G/A	38.9	intron
rs17779352	7:17310002	T/C	8.1	exon, synonymous variant
rs1476080	7:17318249	C/A	37.2	intron, regulatory region variant
rs2237297	7:17319970	C/T	9.3	intron
rs17137566	7:17320897	T/C	15.7	intron
rs4236290	7:17323944	T/C	12.0	intron
rs6960165	7:17328461	A/G	24.1	intron
rs2158041	7:17328796	G/A	23.8	intron
rs3802082	7:17330557	T/A	15.3	intron
rs7811989	7:17331739	G/A	27.3	intron
rs2066853	7:17339486	G/A	9.8	exon, missense variant
rs2040623	7:17341038	T/G	19.4	intron
rs2106728	7:17342116	A/G	35.2	intron

Table 3.3: GEE Regression models of 1976 log10TCDD exposure on child birthweight stratified by genotype at each locus using dominant model of inheritance, SWHS, 1996-2014

		0 minor alleles			1 or 2 minor alleles				
SNP	**Genetic Model**	**β_{adj} (95%CI)**		**p**	**β_{adj} (95%CI)**		**p**	**p_{int}**	**$p_{int\text{-}FDR}$**
rs6968865	AA + TA vs TT	21.47	(-68.38, 111.33)	0.64	-60.01	(-122.51, 2.48)	0.06	0.03	0.14
rs3757824	GG + AG vs AA	17.66	(-46.06, 81.37)	0.59	-124.94	(-210.74, -39.13)	0.004**	0.02	0.13
rs10249788	TT + CT vs CC	-1.94	(-60.02, 56.13)	0.95	-136.17	(-244.72, -27.62)	0.01*	0.02	0.13
rs713150	GG + CG vs CC	-44.19	(-117.31, 28.92)	0.24	-1.51	(-77.27, 74.24)	0.97	0.78	0.86
rs17722841	AA + GA vs GG	-50.43	(-109.22, 8.36)	0.09	-2.31	(-108.75, 104.14)	0.97	0.72	0.86
rs2282885	CC + TC vs TT	-88.56	(-165.88, -11.24)	0.02*	13.62	(-55.48, 82.73)	0.70	0.07	0.22
rs3802083	AA + GA vs GG	-37.45	(-132.47, 57.58)	0.44	-32.45	(-93.75, 28.86)	0.30	0.66	0.84
rs17779352	CC + TC vs TT	-36.05	(-89.62, 17.53)	0.19	-5.98	(-171.12, 159.16)	0.94	0.59	0.80
rs1476080	AA + CA vs CC	-14.83	(-104.58, 74.92)	0.75	-44.12	(-107.23, 18.99)	0.17	0.37	0.67
rs2237297	TT + CT vs CC	-23.44	(-80.60, 33.72)	0.42	-53.89	(-171.96, 64.18)	0.37	0.42	0.67
rs17137566	CC + TC vs TT	-14.37	(-76.53, 47.78)	0.65	-77.53	(-168.41, 13.35)	0.09	0.42	0.67
rs4236290	CC + TC vs TT	-8.35	(-67.01, 50.31)	0.78	-105.1	(-211.57, 1.38)	0.05	0.14	0.38
rs6960165	GG + AG vs AA	-45.86	(-116.46, 24.74)	0.20	-9.52	(-83.73, 64.69)	0.80	0.81	0.86
rs2158041	AA + GA vs GG	-45.03	(-115.55, 25.49)	0.21	-16.18	(-90.66, 58.30)	0.67	0.90	0.90
rs3802082	AA + TA vs TT	4.28	(-59.40, 67.96)	0.90	-80.9	(-169.58, 7.79)	0.07	0.23	0.55
rs7811989	AA + GA vs GG	-17.22	(-91.45, 57.01)	0.65	-46.50	(-117.87, 24.87)	0.20	0.34	0.67
rs2066853	AA + GA vs GG	-26.61	(-83.39, 30.16)	0.36	-34.27	(-156.09, 87.55)	0.58	0.47	0.69
rs2040623	GG + TG vs TT	36.35	(-29.46, 102.16)	0.28	-113.38	(-196.58, -30.18)	0.01**	0.02	0.13
rs2106728	GG + AG vs AA	-82.11	(-159.84, -4.38)	0.04*	9.98	(-58.90, 78.86)	0.78	0.07	0.22

Models adjusted for gestational age (weeks), history of low birthweight, maternal age at pregnancy, parity, maternal height, birth year, child sex. $P_{interaction}$ from model with an interaction term between log10TCDD and genotype.

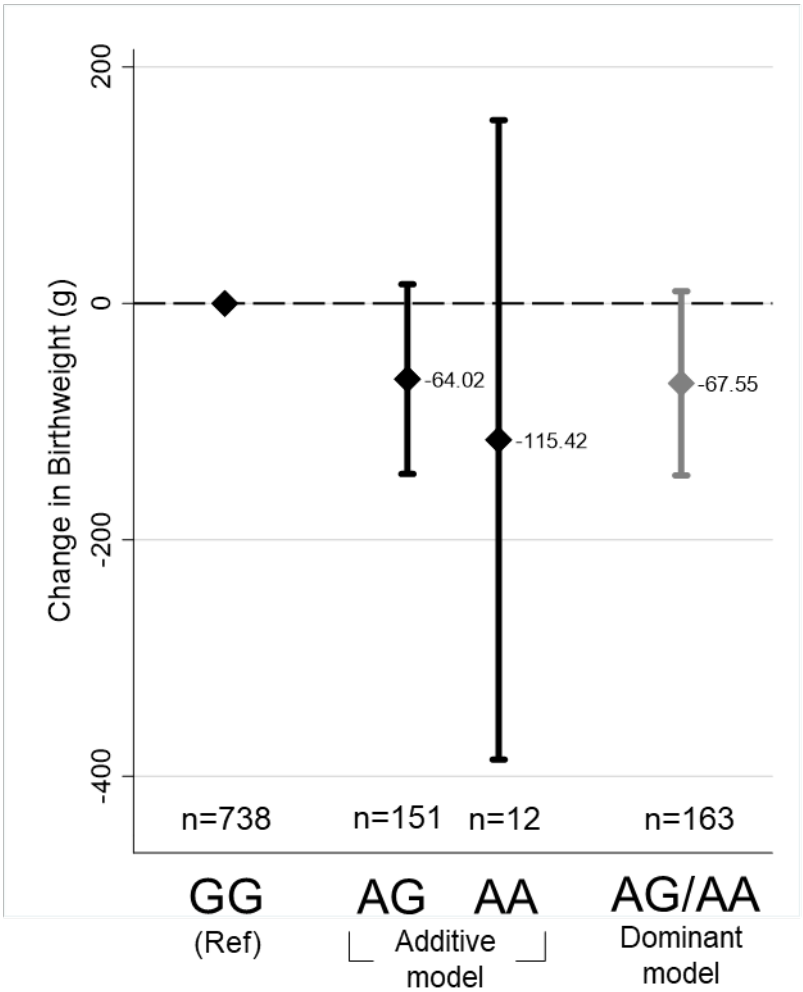
* p-value significant at alpha <0.05

** p-value significant at alpha <0.01

3.9 FIGURES

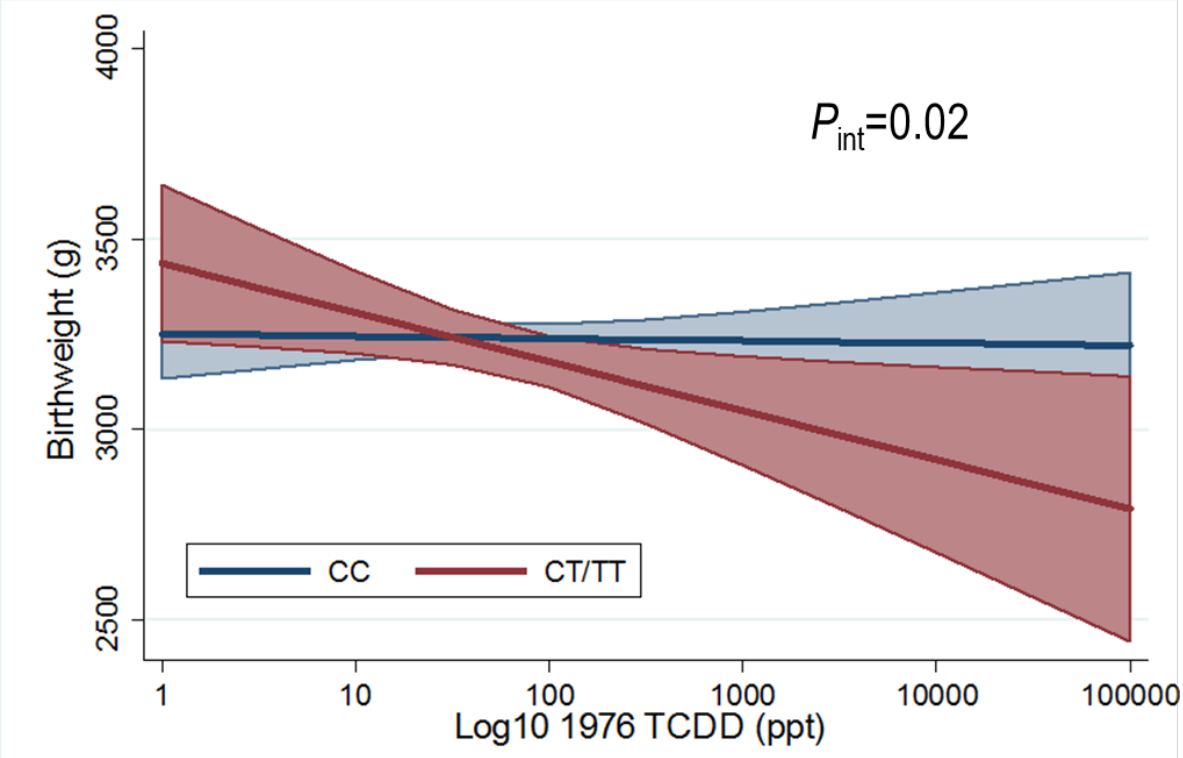
Tables 3.1-3.5: AHR Gene-Dioxin Interactions and Birthweight in the Seveso Second Generation Health Study

Figure 3.1: Main effects of maternal AHR SNP rs2066853 on birthweight under dominant and additive genetic models, SWHS, 1996-2014



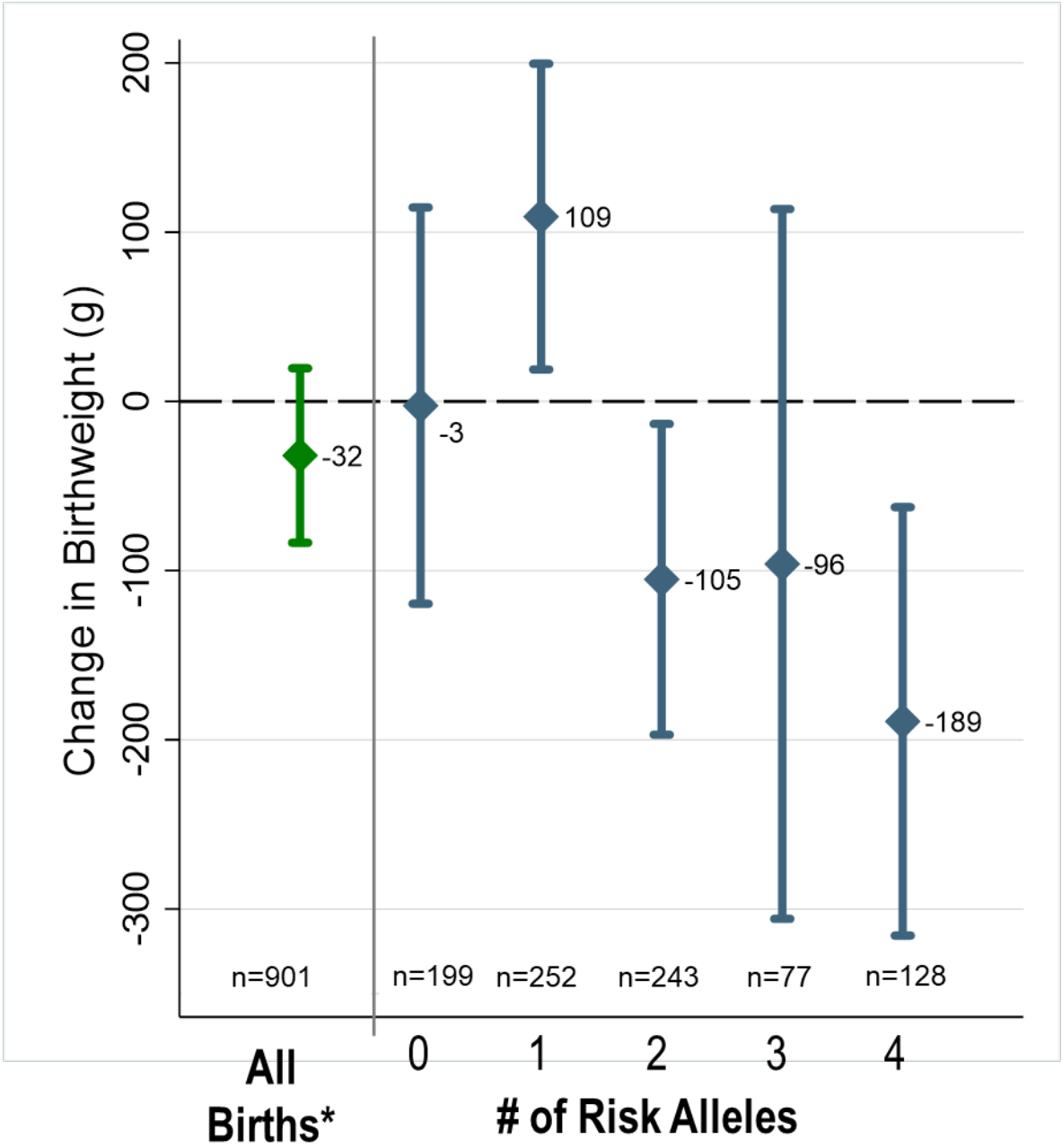
Models adjusted for gestational age (weeks), history of low birthweight, maternal age at pregnancy, parity, maternal height, birth year, child sex

Figure 3.2: Interactive effects of maternal rs10249788*Maternal log10TCDD on birthweight under a dominant genetic model, SWHS, 1996-2014



Models adjusted for gestational age (weeks), history of low birthweight, maternal age at pregnancy, parity, maternal height, birth year, child sex.

Figure 3.3: Association of maternal TCDD levels on birthweight, stratified by maternal risk allele score. Score based on 4 SNPs in *AHR*, SWHS, 1996-2014



Models adjusted for gestational age (weeks), history of low birthweight, maternal age at pregnancy, parity, maternal height, birth year, child sex.
* Model without consideration of risk allele score.

3.10 SUPPLEMENTARY MATERIALS

3.10.1 Supplementary Tables

Tables 3.1-3.5: AHR Gene-Dioxin Interactions and Birthweight in the Seveso Second Generation Health Study

Supplementary Table 3.1: GEE Regression models of main effects between maternal SNPs and maternal 1976 log10TCDD exposure on child birthweight using dominant model of inheritance, SWHS, 1996-2014

				TCDD[a]		SNP[a]	
SNP	**Genetic Model**	**Live births**	**Unique mothers**	**βadj (95%CI)**		**βadj (95%CI)**	
rs6968865	AA + TA vs TT	901	562	-32.03	(-83.59, 19.52)	-26.09	(-90.19, 38.02)
rs3757824	GG + AG vs AA	899	561	-32.50	(-84.28, 19.28)	8.33	(-55.80, 72.45)
rs10249788	TT + CT vs CC	901	562	-32.67	(-84.29, 18.94)	-30.73	(-102.41, 40.96)
rs713150	GG + CG vs CC	884	551	-22.36	(-75.11, 30.39)	-8.65	(-70.46, 53.17)
rs17722841	AA + GA vs GG	899	561	-33.99	(-85.77, 17.78)	-40.40	(-108.65, 27.85)
rs2282885	CC + TC vs TT	901	562	-31.79	(-83.36, 19.77)	-6.18	(-67.88, 55.53)
rs3802083	AA + GA vs GG	901	562	-31.76	(-83.22, 19.71)	-47.41	(-111.09, 16.27)
rs17779352	CC + TC vs TT	901	562	-31.45	(-82.89, 20.00)	40.13	(-45.40, 125.65)
rs1476080	AA + CA vs CC	899	561	-32.83	(-84.34, 18.68)	-35.39	(-97.82, 27.04)
rs2237297	TT + CT vs CC	899	561	-31.04	(-82.57, 20.49)	-58.55	(-138.21, 21.10)
rs17137566	CC + TC vs TT	901	562	-32.28	(-83.83, 19.27)	-18.08	(-84.92, 48.75)
rs4236290	CC + TC vs TT	901	562	-32.03	(-83.61, 19.54)	-2.25	(-75.69, 71.18)
rs6960165	GG + AG vs AA	899	561	-29.70	(-81.24, 21.85)	0.27	(-61.37, 61.92)
rs2158041	AA + GA vs GG	901	562	-31.98	(-83.52, 19.56)	0.45	(-61.21, 62.10)
rs3802082	AA + TA vs TT	888	554	-21.58	(-73.67, 30.50)	0.55	(-67.04, 68.13)
rs7811989	AA + GA vs GG	901	562	-31.95	(-83.51, 19.62)	1.57	(-59.58, 62.72)
rs2066853	AA + GA vs GG	901	562	-30.91	(-82.41, 20.58)	-67.55	(-145.46, 10.36)*
rs2040623	GG + TG vs TT	895	558	-20.22	(-72.47, 32.03)	3.13	(-60.62, 66.88)
rs2106728	GG + AG vs AA	901	562	-31.41	(-83.00, 20.18)	-13.23	(-75.00, 48.54)

Models adjusted for gestational age (weeks), history of low birthweight, maternal age at pregnancy, pre-explosion parity, maternal height, birth year, child sex. [a] Models without GxE interaction term. *p-value <0.1

Supplementary Table 3.2: Crude GEE Regression models of interaction between maternal SNPs and maternal 1976 log10TCDD exposure on child birthweight using dominant model of inheritance, SWHS, 1996-2014

		0 minor alleles		1 or 2 minor alleles		
SNP	**Genetic Model**	**β_{adj} (95%CI)**		**β_{adj} (95%CI)**		**p_{int}**
rs6968865	AA + TA vs TT	25.18	(-77.07, 127.43)	-84.32	(-160.11, -8.53)*	0.10
rs3757824	GG + AG vs AA	-6.11	(-81.26, 69.05)	-138.71	(-243.57, -33.85)**	0.05
rs10249788	TT + CT vs CC	-12.81	(-81.04, 55.41)	-175.85	(-311.64, -40.06)*	0.03
rs713150	GG + CG vs CC	-77.44	(-159.80, 4.91)	-11.19	(-106.68, 84.31)	0.30
rs17722841	AA + GA vs GG	-62.03	(-133.23, 9.16)	-27.00	(-149.33, 95.33)	0.59
rs2282885	CC + TC vs TT	-81.99	(-182.77, 18.78)	-22.50	(-98.25, 53.25)	0.36
rs3802083	AA + GA vs GG	-72.11	(-175.84, 31.62)	-38.50	(-114.14, 37.14)	0.61
rs17779352	CC + TC vs TT	-45.89	(-109.99, 18.22)	-52.71	(-242.04, 136.62)	0.86
rs1476080	AA + CA vs CC	-41.44	(-138.85, 55.97)	-52.40	(-131.10, 26.30)	0.87
rs2237297	TT + CT vs CC	-34.11	(-99.88, 31.66)	-113.83	(-274.03, 46.37)	0.36
rs17137566	CC + TC vs TT	-33.84	(-105.80, 38.11)	-86.72	(-201.82, 28.38)	0.46
rs4236290	CC + TC vs TT	-35.31	(-105.83, 35.21)	-96.54	(-217.79, 24.71)	0.40
rs6960165	GG + AG vs AA	-78.64	(-157.65, 0.36)	-10.67	(-106.45, 85.11)	0.28
rs2158041	AA + GA vs GG	-78.01	(-156.90, 0.87)	-13.53	(-109.46, 82.41)	0.31
rs3802082	AA + TA vs TT	-19.34	(-93.23, 54.54)	-97.64	(-209.83, 14.55)	0.26
rs7811989	AA + GA vs GG	-52.25	(-139.50, 35.00)	-42.15	(-128.13, 43.84)	0.89
rs2066853	AA + GA vs GG	-31.73	(-96.56, 33.09)	-118.83	(-284.61, 46.96)	0.30
rs2040623	GG + TG vs TT	-4.93	(-81.46, 71.60)	-114.04	(-216.63, -11.45)*	0.10
rs2106728	GG + AG vs AA	-86.55	(-184.70, 11.60)	-18.21	(-95.96, 59.54)	0.28

* p-value <0.05

** p-value <0.01

Supplementary Table 3.3: GEE Regression models of 1976 log10TCDD exposure on child birthweight stratified by genotype at each locus using dominant model of inheritance, excluding preterm births, SWHS, 1996-2014

		0 minor alleles		1 or 2 minor alleles		
SNP	**Genetic Model**	**βadj (95%CI)**		**βadj (95%CI)**		**p_{int}**
rs6968865	AA + TA vs TT	10.29	(-82.29, 102.88)	-55.08	(-116.04, 5.89)	0.06
rs3757824	GG + AG vs AA	19.55	(-45.20, 84.30)	-121.87	(-205.85, -37.89)**	0.03
rs10249788	TT + CT vs CC	-0.55	(-59.69, 58.59)	-130.55	(-230.94, -30.17)*	0.02
rs713150	GG + CG vs CC	-38.72	(-112.68, 35.25)	-0.34	(-75.96, 75.28)	0.94
rs17722841	AA + GA vs GG	-53.81	(-111.86, 4.23)	14.22	(-92.82, 121.26)	0.42
rs2282885	CC + TC vs TT	-87.68	(-165.29, -10.07)*	13.83	(-54.94, 82.60)	0.06
rs3802083	AA + GA vs GG	-32.00	(-131.01, 67.01)	-31.88	(-91.60, 27.84)	0.75
rs17779352	CC + TC vs TT	-35.81	(-88.67, 17.05)	-3.40	(-180.31, 173.51)	0.59
rs1476080	AA + CA vs CC	-9.65	(-102.19, 82.90)	-44.61	(-105.97, 16.76)	0.40
rs2237297	TT + CT vs CC	-20.87	(-78.81, 37.07)	-75.88	(-184.30, 32.55)	0.32
rs17137566	CC + TC vs TT	-11.24	(-74.11, 51.62)	-74.89	(-164.56, 14.77)	0.37
rs4236290	CC + TC vs TT	-3.31	(-62.88, 56.25)	-119.75	(-220.25, -19.26)*	0.08
rs6960165	GG + AG vs AA	-40.78	(-111.76, 30.20)	-11.84	(-86.25, 62.56)	0.98
rs2158041	AA + GA vs GG	-40.98	(-111.95, 29.99)	-15.88	(-90.36, 58.60)	0.99
rs3802082	AA + TA vs TT	5.02	(-59.07, 69.11)	-72.12	(-160.50, 16.25)	0.23
rs7811989	AA + GA vs GG	-10.55	(-86.23, 65.14)	-49.42	(-119.73, 20.88)	0.34
rs2066853	AA + GA vs GG	-23.84	(-81.44, 33.76)	-60.46	(-172.00, 51.08)	0.37
rs2040623	GG + TG vs TT	39.38	(-28.13, 106.90)	-115.04	(-194.00, -36.07)**	0.02
rs2106728	GG + AG vs AA	-87.86	(-165.45, -10.27)*	15.59	(-53.24, 84.42)	0.05

Models adjusted for gestational age (weeks), history of low birthweight, maternal age at pregnancy, parity, maternal height, birth year, child sex. $P_{interaction}$ from model with an interaction term between log10TCDD and genotype.

* p-value <0.05

** p-value <0.01

Supplementary Table 3.4: GEE Regression models of interaction between maternal SNPs and maternal 1976 log10TCDD exposure on child birthweight using dominant model of inheritance among all births, SWHS, 1996-2014

		0 minor alleles		1 or 2 minor alleles		
SNP	**Genetic Model**	**β_{adj} (95%CI)**		**β_{adj} (95%CI)**		**p_{int}**
rs6968865	AA + TA vs TT	38.16	(-57.89, 134.20)	-51.92	(-114.83, 11.00)	0.02
rs3757824	GG + AG vs AA	23.13	(-42.84, 89.11)	-107.92	(-195.60, -20.25)*	0.04
rs10249788	TT + CT vs CC	2.22	(-57.76, 62.20)	-108.55	(-220.33, 3.24)	0.05
rs713150	GG + CG vs CC	-20.73	(-97.96, 56.50)	1.65	(-73.64, 76.95)	0.89
rs17722841	AA + GA vs GG	-36.97	(-98.15, 24.21)	2.35	(-103.99, 108.69)	0.83
rs2282885	CC + TC vs TT	-77.04	(-156.21, 2.14)	23.46	(-47.54, 94.45)	0.10
rs3802083	AA + GA vs GG	-14.82	(-116.22, 86.58)	-24.77	(-86.19, 36.65)	0.47
rs17779352	CC + TC vs TT	-30.09	(-84.82, 24.65)	40.08	(-135.22, 215.38)	0.30
rs1476080	AA + CA vs CC	3.60	(-91.36, 98.56)	-35.60	(-98.85, 27.65)	0.28
rs2237297	TT + CT vs CC	-12.10	(-71.30, 47.10)	-47.94	(-164.34, 68.46)	0.37
rs17137566	CC + TC vs TT	-0.04	(-64.61, 64.52)	-71.57	(-162.23, 19.10)	0.32
rs4236290	CC + TC vs TT	-0.31	(-60.53, 59.91)	-78.68	(-189.67, 32.31)	0.23
rs6960165	GG + AG vs AA	-25.71	(-100.15, 48.73)	-6.97	(-80.78, 66.84)	0.88
rs2158041	AA + GA vs GG	-24.35	(-98.70, 50.01)	-13.79	(-87.86, 60.28)	0.78
rs3802082	AA + TA vs TT	19.34	(-46.64, 85.32)	-73.37	(-162.20, 15.46)	0.17
rs7811989	AA + GA vs GG	-0.37	(-78.02, 77.28)	-39.20	(-111.07, 32.67)	0.28
rs2066853	AA + GA vs GG	-15.83	(-74.57, 42.90)	-23.81	(-144.46, 96.84)	0.42
rs2040623	GG + TG vs TT	44.40	(-23.63, 112.44)	-99.98	(-184.72, -15.23)*	0.03
rs2106728	GG + AG vs AA	-70.87	(-150.38, 8.65)	20.00	(-50.85, 90.86)	0.09

Models adjusted for gestational age (weeks), history of low birthweight, maternal age at pregnancy, parity, maternal height, birth year, child sex. $P_{interaction}$ from model with an interaction term between log10TCDD and genotype.

* p-value <0.05

** p-value <0.01

Supplementary Table 3.5: Robust regression models of interaction between maternal SNPs and maternal 1976 log10TCDD exposure on child birthweight using dominant model of inheritance among first births post-explosion, SWHS, 1996-2014

		0 minor alleles		1 or 2 minor alleles		
SNP	**Genetic Model**	**β_{adj} (95%CI)**		**β_{adj} (95%CI)**		**p_{int}**
rs6968865	AA + TA vs TT	-27.07	(-134.52, 80.37)	-60.66	(-131.62, 10.30)	0.16
rs3757824	GG + AG vs AA	-6.79	(-83.86, 70.27)	-124.62	(-211.37, -37.88)**	0.07
rs10249788	TT + CT vs CC	-30.02	(-97.77, 37.73)	-117.25	(-224.79, -9.71)*	0.10
rs713150	GG + CG vs CC	-52.40	(-131.48, 26.68)	-14.34	(-93.06, 64.37)	0.90
rs17722841	AA + GA vs GG	-43.68	(-111.83, 24.48)	-55.89	(-177.41, 65.63)	0.73
rs2282885	CC + TC vs TT	-79.15	(-172.16, 13.86)	-22.59	(-95.25, 50.06)	0.27
rs3802083	AA + GA vs GG	-88.88	(-196.55, 18.79)	-35.30	(-105.94, 35.33)	0.84
rs17779352	CC + TC vs TT	-44.70	(-105.54, 16.14)	-25.55	(-208.75, 157.65)	0.59
rs1476080	AA + CA vs CC	-63.86	(-163.20, 35.48)	-39.64	(-111.33, 32.05)	0.80
rs2237297	TT + CT vs CC	-40.12	(-106.70, 26.46)	-65.21	(-181.31, 50.88)	0.64
rs17137566	CC + TC vs TT	-27.17	(-101.91, 47.57)	-92.19	(-183.45, -0.93)*	0.49
rs4236290	CC + TC vs TT	-33.89	(-95.73, 27.96)	-75.61	(-210.58, 59.35)	0.45
rs6960165	GG + AG vs AA	-57.34	(-132.41, 17.72)	-23.86	(-112.19, 64.46)	0.95
rs2158041	AA + GA vs GG	-56.77	(-131.77, 18.23)	-31.45	(-120.16, 57.26)	0.86
rs3802082	AA + TA vs TT	-7.75	(-76.90, 61.41)	-90.37	(-181.08, 0.35)	0.27
rs7811989	AA + GA vs GG	-43.16	(-124.02, 37.69)	-46.51	(-129.26, 36.23)	0.45
rs2066853	AA + GA vs GG	-44.60	(-110.84, 21.64)	-32.22	(-155.04, 90.60)	0.88
rs2040623	GG + TG vs TT	14.36	(-58.05, 86.77)	-107.75	(-188.08, -27.43)**	0.07
rs2106728	GG + AG vs AA	-76.51	(-170.44, 17.41)	-21.18	(-93.78, 51.41)	0.23

Models adjusted for gestational age (weeks), history of low birthweight, maternal age at pregnancy, parity, maternal height, birth year, child sex. $P_{interaction}$ from model with an interaction term between log10TCDD and genotype.

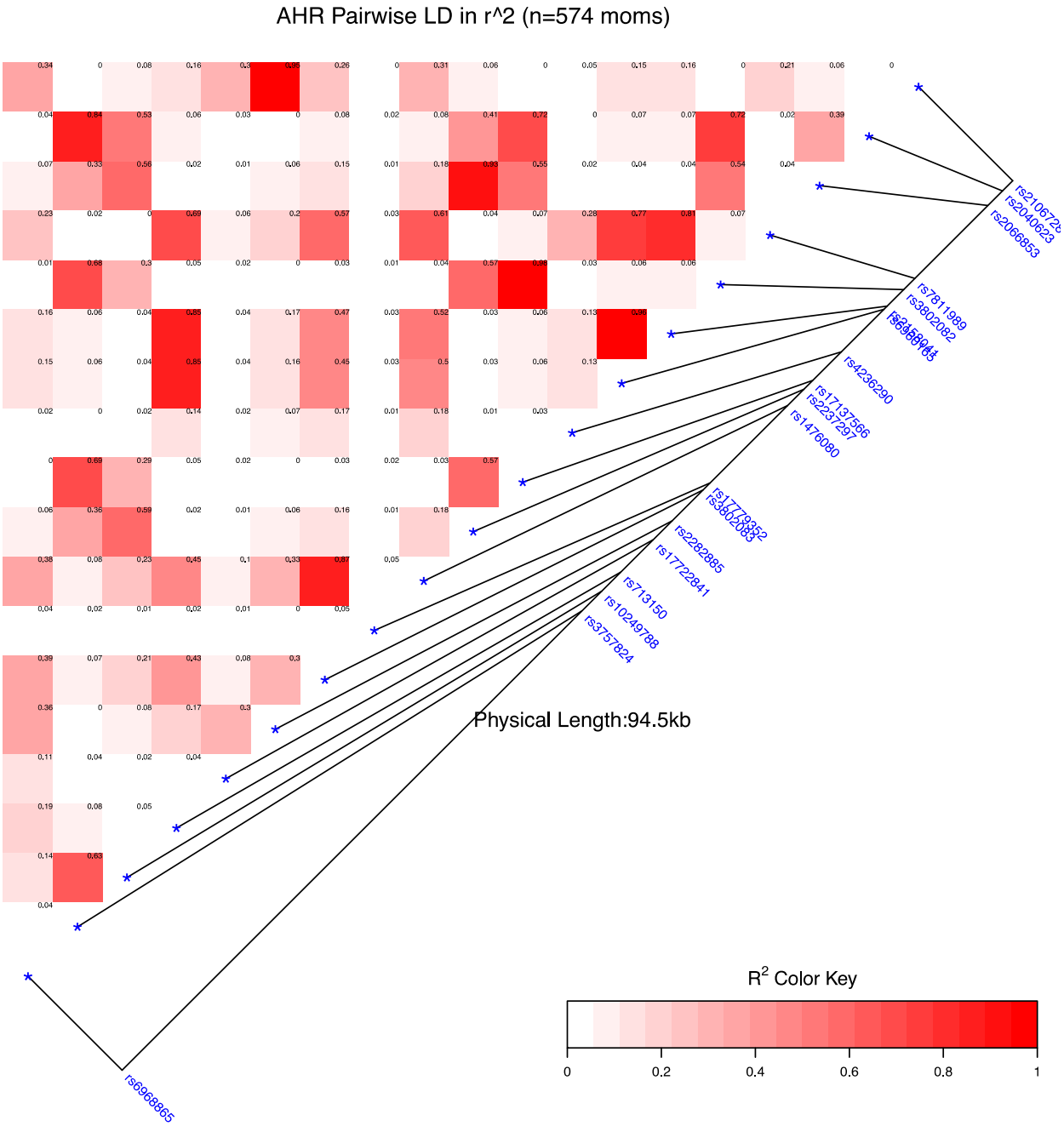
* p-value <0.05

** p-value <0.01

3.10.2 Supplementary Figures

Tables 3.1-3.5: AHR Gene-Dioxin Interactions and Birthweight in the Seveso Second Generation Health Study

Supplementary Figure 3.1: Linkage Disequilibrium heat map of 19 SNPs in *AHR*, SWHS, 1996-2014. SNPs are ordered by their chromosomal position and relative distances from each other across the gene. The beginning of the gene runs from left to right.



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4. CONCLUSIONS

4.1 SYNTHESIS OF FINDINGS

With several high profile industrial accidents, Agent Orange, and Love Canal on its record, dioxin and dioxin-like compounds are repeat offenders in the history of environmental health. Their most toxic congener, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), is a persistent organic pollutant known to cause carcinogenic and endocrine-disrupting effects[1]. Given their historical notoriety, industrially-produced dioxins are tightly controlled In the United States by EPA's authorizing statutes within the Clean Air Act, Clean Water Act, Safe Drinking Water Act, and Toxic Substance Control Act and they are regularly monitored in the food supplies of other countries.[2] As a result of these regulatory and voluntary actions by EPA and industry, respectively, dioxin levels in humans and the environment have dropped substantially in the last few decades. Nevertheless, many urban and natural processes such as fuel combustion, trash burning, and forest fires continue to release dioxins into the environment[3]. Thus, the ubiquitous presence of dioxin in the food supply, air, breast milk, and maternal blood continues to pose a risk to human health. Further, dioxin's relatively long half-life in the body (4-10 years in adults) and mounting epidemiological and animal evidence suggesting that its toxicity may persist across generations through epigenetic changes[4-8], warrant a sustained investigation of its effects on human health.

Chapters 1 and 2 provided the first studies of TCDD's exclusive effects on neurodevelopment in women and children. While the findings in **chapter 1** generally indicate no adverse association between postnatal TCDD exposure and cognition and physical functioning in women, the SWHS were relatively young at the age of assessment and the possibility remains open that their exposures could influence later-life neurological decline and/or dementia. The impact of neurotoxicants, particularly those with endocrine-disrupting properties, on the aging brain have not been widely explored in humans. Future phases in the SWHS may explore Alzheimer's, dementia, and other neurological conditions associated with aging, drawing attention to these understudied windows of susceptibility in later life. With improved longevity across the industrializing and industrialized world, elder health is becoming a major priority. Research on the interplay of past and current environmental exposures on the aging brain and body will have important socioeconomic implications for promoting geriatric healthcare and quality of life.

In **chapter 2**, we observed primarily null associations between maternal TCDD exposures and the neuropsychological functioning of their 7-17 year old children. However, there was suggestive evidence that child sex and lactational histories could modify sensitivity to prenatal TCDD exposure, particularly with respect to psychometric domains of learning and attention. However, the story is far from clear and warrants replication in larger studies though few populations have exposures as high as Seveso. One possibility is to assess the older children in the second generation who were born closer to the time of the accident and consequently may have received a higher prenatal exposure than the children in our sample. This study on the 7-17 year old offspring, though difficult to interpret in isolation, is an important first step and identifies paths of future investigation of the neuropsychological health in the second generation and other cohorts.

Chapter 3 offered a particularly noteworthy finding that maternal genetics may modify the association between the SWHS mother's 1976 exposure and her child's birthweight. The

variants with evidence of interaction with TCDD were located in regions of the *AHR* gene that likely contribute to gene regulation, and, consequently, could plausibly influence variability in toxic response to TCDD exposure. Furthermore, these implicated SNPs, when examined in an additive risk allele score, also appeared to cumulatively modify the relationship between TCDD and birthweight. To follow up on this finding, future analyses will examine how *AHR* genetics influence apparent TCDD elimination half-lives in a subset of SWHS women for whom TCDD was measured in serum at two time points (1976 after the accident and in 1996 at study enrollment). Investigations of the child's *AHR* genetics are also planned.

This dissertation, in its examination of factors that shape human sensitivity to TCDD, continues the objective of the SWHS to understand TCDD's far-ranging effects on disease etiology. In particular, this work elucidates susceptible subgroups defined by genetics, sex, lactational history, and windows of development, underlining the reality that environmental toxicants that we are exposed to today may have important implications for our future health as well as the health of our children and grandchildren. The results of this study, in conjunction with future work in the SWHS and other special exposure studies, could meaningfully inform further research, policy, and regulation of endocrine disrupting compounds. Furthermore, our findings pertaining to genetic susceptibility could shed light on toxicological mechanisms of PCBs, PAHs, and other AhR-inducing toxic compounds, possibly identifying susceptible groups for future targeted therapies or interventions.

This research sought to better understand the societal consequences of environmental health calamities on long-term public health. Its particular focus on susceptible subpopulations of women and their children draws attention to the health and economic burdens these exposures place on future generations and the challenges of environmental health sustainability. These analyses build upon the unique design and history of the SWHS while striving to remain acutely aware and sensitive to the motivations underlying our participants' enrollment. This work would not be possible without the generosity and selflessness of these women; in return, science and society have an obligation to learn as much as we can from the Seveso accident and prevent similar tragedies from occurring in the future.

4.2 FUTURE DIRECTIONS:

In summary, the findings from this dissertation lay the foundation for several key future studies:

- Given that the SWHS were relatively young at the physical and neuropsychological assessments in 1996 and 2008, their continued follow-up into old age is warranted. Future assessments should consider additional neuropsychological outcomes, including psychomotor tasks. Grip strength should also be re-evaluated at older ages.
- Further studies of the neuropsychological health of the second generation should also include the older children in the cohort who possibly received the highest *in utero* exposures to TCDD.
- Future research examining the relationship between prenatal exposure to endocrine-disrupting compounds and neurodevelopment should focus on effect modification by child sex and lactation history.
- The mechanisms by which polymorphisms in AHR influence dioxin susceptibility, while biologically plausible, need to be confirmed in future molecular work.

- Whether child genetics also contribute to the relationship between prenatal dioxin exposure and fetal growth will be investigated in future work. Very few studies have considered the interplay between maternal and child genetics on fetal sensitivity to environmental chemicals.
- We will also consider how variation in additional genes within the canonical AhR-pathway including CYP1A1, CYP1A2, CYP1B1, AHRR, and ARNT, in interaction with TCDD exposure, influence health outcomes in both the SWHS and their children.

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