The Molecular Epidemiology of Dioxin Exposure in Former Chemical Workers

BY

PETER RUESTOW
BA, University of Michigan, 2004
MPH, University of Michigan, 2006

THESIS

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Defense Committee:

Dan Hryhorczuk, MD, MPH, FACMT—Chair
Irina Dardynskaia, MD, PhD—Environmental and Occupational Health Sciences
Lee Friedman, PhD—Environmental and Occupational Health Sciences
Peter Gann, MD, ScD—Pathology
Leslie Stayner, PhD, MS—Epidemiology

DEDICATION

This work is dedicated to the individuals who participated in this study—I believe that, together, we have made a meaningful contribution to the field of occupational health and safety.

I would not be in the position to present these findings if not for the unwavering support of my family and friends, including my darling wife. Much of what I do, I do to make you proud.

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LIST OF ABBREVIATIONS

ΔCt	Normalized Cycles to Threshold
2,4-D	2,4-dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
AhR	Aryl Hydrocarbon Receptor
AhRR	Aryl Hydrocarbon Receptor Repressor
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
BaP	Benzo(a)pyrene
BCL2	B-cell Leukemia/Lymphoma 2
bHLH	Basic Helix-Loop-Helix
BMI	Body Mass Index
CDC	Centers for Disease Control and Prevention
CI	Confidence Interval
Ct	Cycles to Threshold
CYP1A1	Cytochrome P450, Family 1, Subfamily A, Polypeptide 1
CYP1A2	Cytochrome P450, Family 1, Subfamily A, Polypeptide 2
CYP1B1	Cytochrome P450, Family 1, Subfamily B, Polypeptide 1
DL-PCB	Dioxin-Like Polychlorinated Biphenyl
DNA	Deoxyribonucleic acid
DRE	Dioxin Response Element
ED_{50}	Half Maximal Effective Dose
EDTA	Ethylenediaminetetraacetic acid
FDR	False Discovery Rate
Hsp90	Heat Shock Protein 90

LIST OF ABBREVIATIONS (continued)

IgH	Immunoglobulin Heavy Chain
LOD	Limit of Detection
mL	Milliliter
mRNA	Messenger Ribonucleic Acid
NDL-PCB	Non-Dioxin-Like Polychlorinated Biphenyl
NHANES	National Health and Nutrition Examination Survey
NHL	Non-Hodgkin's Lymphoma
NIOSH	National Institute for Occupational Safety and Health
NRF2	Nuclear Factor Erythroid 2-Related Factor 2
PAH	Polycyclic Aromatic Hydrocarbon
PAS	Period-Aryl Hydrocarbon Nuclear Translocator-Simple-Minded Domain
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCB	Polychlorinated Biphenyl
PCB 126	3,3',4,4',5-Pentachlorobiphenyl
PCB 153	2,2',3,4,4',5,5'-Heptachlorobiphenyl
PCB 180	2,2',4,4',5,5'-Hexachlorobiphenyl
PCDD	Polychlorinated Dibenzo-P-Dioxin
PCDF	Polychlorinated Dibenzofuran
PCR	Polymerase Chain Reaction
PPM	Parts per million
PPT	Parts per Trillion
qPCR	Quantitative Polymerase Chain Reaction

LIST OF ABBREVIATIONS (continued)

RNA	Ribonucleic acid
RR	Relative Risk
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TCDD	2,3,7,8-Tetrachlorodibenzo-P-Dioxin
TCP	Trichlorophenol
TCP-Cu	Trichlorophenol Copper Salt
TEF	Toxic Equivalency Factor
TEQ	Total Toxic Equivalency
UIC	University of Illinois at Chicago
WHO	World Health Organization
XAP2	Immunophilin-like Protein Hepatitis B Virus X-associated Protein 2

SUMMARY

The major aim of the studies described in detail herein was to further characterize the mechanisms through which dioxins exert their toxic effects in humans. A large international multidisciplinary research team enrolled, surveyed, and examined two cohorts of Eastern European subjects. One was composed of chemical factory workers occupationally exposed to dioxins. The other was composed of residents from a nearby city without known dioxin exposure. Preliminary analyses revealed relatively high levels of dioxin-like (DL) and non-dioxin-like (NDL) polychlorinated biphenyls (PCBs) in this population-based group.

An association between dioxin exposure and Non-Hodgkin's Lymphoma (NHL) has been demonstrated in other occupationally and environmentally exposed cohorts, though the molecular mechanisms explaining this relationship are debated. A potential link between dioxin exposure and the presence of t(14;18) chromosomal translocations in lymphocytes, a likely intermediate step in the causal pathway for two NHL subtypes, has also been established. Our study of risk factors for t(14;18) expansion demonstrated a significant association between closer-to-background serum levels of DL PCB 126 and t(14;18) frequency in the environmentally exposed cohort, and this relationship was modified by smoking habit. Risk was increased in current (RR=1.51, 95%CI: 1.17–1.94) and former (RR=1.13, 95%CI: 1.03–1.24) smokers, but not in never smokers (RR=.99, 95%CI: 0.95–1.03). These findings suggest a biologically plausible mechanism for increased risk of NHL.

Dioxin toxicity is believed to occur as a result of persistent activation of the aryl hydrocarbon receptor (AhR), a transcription factor that regulates several drug-metabolizing genes, among others. We investigated the relationship between dioxin exposure and gene expression in a subsample of the two cohorts. Expression of the AhR itself was found to be significantly associated with serum dioxin levels, with expression in the highest tertile of dioxin dose 1.4-fold higher than that in the lowest tertile. We

further demonstrated the antagonist activity of NDL-PCB 153 on dioxin-induced AhR gene expression. This is the first time that this chemical interaction has been reported in a human observational study.

One of the well-established health effects of dioxin exposure (and subsequent AhR activation) is chloracne, a condition characterized by skin lesions resembling acne vulgaris. In an attempt to identify risk factors that might explain inter-individual differences in chloracne development after dioxin exposure, we found a much higher prevalence of smoking in those who developed chloracne versus those who did not in the occupationally exposed cohort. Given recent findings regarding the role of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway in chloracne development, we suggest possible mechanisms through which risk of chloracne may be modified by concurrent 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and cigarette constituent exposures.

The results of these studies have important implications for risk assessment when it comes to dioxin and PCB exposure. Highlighted are exciting areas for future research into the molecular toxicity of these chemicals. Furthermore, our findings suggest that risks associated with dioxin toxicity may be significantly altered by avoidance or cessation of smoking, a modifiable behavior.

I. BACKROUND AND SIGNIFICANCE

A. Dioxins

1. Properties

The term “dioxin” refers to a class of chemical contaminants that are similar in chemical structure and mode of action. Included in this group of substances are the polychlorinated dibenzo-p-dioxins (PCDDs), the polychlorinated dibenzofurans (PCDFs), and a subcategory of DL-PCBs that share the same mode of action. All are classified as halogenated aromatic hydrocarbons. Both PCDDs and PCDFs have a triple-ring structure—two benzene rings connected by an oxygenated ring. The connecting ring contains two oxygen atoms in PCDDs and one oxygen atom in PCDFs. Dioxin-like PCBs are formed by chlorination of a biphenyl molecule (IARC, 1997).

Among those compounds comprising the dioxin family, the most toxic is TCDD. It has been shown to have toxic effects in both animals and humans and has been classified as carcinogenic to humans (IARC, 1997). This toxic compound has a molecular weight of 322, a 305–306°C freezing point, a 445.5°C boiling point, a log octanol-water partition coefficient of 6.8, and a vapor pressure of 1.50×10^{-9} millimeters of mercury at 25°C. It is essentially insoluble in water, though soluble in organic solvents, such as benzene and acetone (NTP, 2011)—its lipophilic structure is an important factor in understanding how it accumulates in the human body.

Many of the effects of TCDD are moderated by the AhR. This mode of action is further elaborated later in this section. Several other PCDDs, PCDFs, and PCBs share an affinity for binding with the AhR, and so are aptly referred to as “dioxin-like.” Currently, these include 7 PCDDs, 10 PCDFs, 4 non-ortho-polychlorinated biphenyls, and 8 mono-ortho-polychlorinated biphenyls. Though the mechanism of action is the same, the toxic potency of each substance is unique. As a result, the World Health Organization (WHO) developed a relative toxic potency ranking scheme, assigning a Toxic Equivalency Factor (TEF) to each compound. The TEF represents the order of magnitude by which the compound’s

toxic potency differs from that of the most toxic compound, TCDD. It typically represents the ratio of TCDD's half maximal effective dose (ED_{50}) to the ED_{50} of the dioxin of interest. As such, the TEF is equal to 1.0 for TCDD and less than 1.0 for all other dioxins (with the exception of 1,2,3,7,8-pentachlorodibenzo-p-dioxin, which also has a TEF of 1.0) (Van den Berg et al., 2006; White and Birnbaum, 2009). The products of the TEF and the concentration of each individual dioxin found in a mixture can be summed to calculate the total toxic equivalency (TEQ) for the mixture (Safe, 1998).

2. **Production**

Dioxins exist as by-products of industrial practices or combustion. They are also produced in the natural environment through processes such as forest fires and volcanic eruption. However, human activities are primarily to blame for the large increase in the generation of these chemicals over the last two centuries. And though many industrial activities responsible for dioxin contamination are now being regulated, other sources, such as uncontrolled burning of household waste in open containers, are still of major concern (White and Birnbaum, 2009). Most evidence for the toxicity of dioxins comes from studies of TCDD (ATSDR, 1998).

Neither PCDDs nor PCDFs were manufactured intentionally, other than for research purposes. However, PCBs were widely produced as cooling fluids in electrical transformers, and they were also used in hydraulic fluids, lubricants, and plasticizers historically (White and Birnbaum, 2009; WHO, 2002). Dioxins are known to be by-products of the chlorine bleaching process used by pulp and paper mills (ATSDR, 1998; Silkworth and Brown, 1996). They are also produced during the incineration of municipal, toxic, and hospital waste; in electrical transformer fires; and in smelters (NTP, 2011). Other important dioxin sources are factories that manufacture chlorophenoxy herbicides (NTP, 2011; Schecter et al., 1997). Used as an herbicide, among other things, 2,4,5-trichlorophenol (TCP) and its derivative 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) were known to have been contaminated with TCDD,

pentachlorodibenzo-p-dioxin, and higher chlorinated dibenzofurans (Ahling et al., 1977; Rappe et al., 1978a). During the Vietnam war, a 50:50 mixture of the N-butyl esters of 2,4,5-T and 2,4-D—code name "Agent Orange"—was used as a defoliant to disrupt enemy ground cover as part of the US military's Operation Ranch Hand. Agent Orange was found to be contaminated with 2–30 parts per million (ppm) TCDD (NTP, 2011; Rappe et al., 1978b).

Relatively low dioxin concentrations have been estimated in cigarette smoke, car exhaust, and household heating systems. Combustion of chlorine-containing products can also produce dioxins. These products include plastics, wood after application of the preservative pentachlorophenol , pesticide wastes, bleached paper, and other chlorinated chemicals (e.g., PCBs) (ATSDR, 1998).

3. Environmental fate and body burden

The environmental fate of dioxins is largely determined by their physical-chemical properties. These include solubility in water, solubility in octanol, and vapor pressure. Experimental and calculated physical-chemical property data for dioxins have been compiled constantly over the years in order to understand and minimize uncertainty around estimates. This research has greatly contributed to our understanding of the distribution and persistence of dioxins in the environment (Aberg et al., 2008; Mackay, 2006).

The physical-chemical properties of dioxins make them a multimedia pollutant—they can exist in atmospheric, terrestrial, or aquatic environmental compartments. As mentioned previously, there are several atmospheric sources of dioxin contamination, including waste incinerators and vehicle exhaust. Dioxins are semi-volatile compounds that can exist in the gaseous phase or be bound to particles in the air (e.g., ash). Lower chlorinated dioxins are more likely to vaporize from particles and travel long distances. In fact, dioxins have been measured in areas with no local sources, in places as remote as the Antarctic. Studies have estimated that 20%–60% of TCDD in the air is in the vapor phase. Dioxins in the

vapor phase can undergo photochemical transformation to less-toxic compounds, but most end up deposited on land or in water (ATSDR, 1998; Buckley-Golder, 1999).

Dioxins can contaminate the terrestrial environment by way of deposition from the atmosphere or application of chlorinated pesticides containing dioxins. Because of strong binding with soil and low water solubility, groundwater contamination through water leaching is unlikely. In the 1960s and 1970s, the use of pesticides contaminated with dioxins, such as 2,4,5-T, may have been a more important source of dioxin contamination than waste incineration. Though certain soil bacteria and fungi can slowly degrade them, dioxins are still able to persist in soil for years. Thus, prior use of pesticides—despite regulatory actions taken over the last few decades—may still be an important exposure source (ATSDR, 1998; Buckley-Golder, 1999).

The aquatic environment can be contaminated by deposition from the atmosphere or through industrial effluent and run-off from soil. The hydrophobic nature of dioxins causes them to quickly partition to organic matter and accumulate in sediments. They may also adhere to microscopic plants and animals, such as plankton. These contaminated particles may be eaten by larger animals, which themselves may be consumed by other predatory animals. In this way, the chemical concentrations at each step of the food chain increase due to the difficulty animals have with breaking down these substances. This process, called "biomagnification," explains why measurable concentrations of dioxins in aquatic animals exist despite undetectable levels in water (ATSDR, 1998; Buckley-Golder, 1999).

There have been numerous studies conducted over the last quarter of a century that aim to describe how dioxins interact with the body using animal models. Most of these have examined the absorption, distribution, metabolism, and elimination of TCDD in particular (IOM, 2009). A brief summary of major findings from these studies follows.

Human exposure to dioxins is believed to occur through the oral, dermal, and inhalation routes. Oral intake allows dioxins into the body, where most make their way from the intestines to the blood

stream. Dioxins enter the lungs via inhalation of contaminated air, though it has been difficult to quantify the proportion that makes it to the blood stream (ATSDR, 1998). Dermal exposure seems to be of less consequence than the other exposure pathways, possibly due to the relatively greater observed resistance to absorption of human skin (Weber et al., 1991). Dermal absorption has also been shown to decrease with increasing dose (Banks and Birnbaum, 1991).

Though absorbed quickly, dioxins are eliminated from the body slowly. Because of this, blood or lipid concentrations of dioxins are thought to be in equilibrium with concentrations in other tissues. Thus, blood or lipid concentrations are considered to be reasonable surrogates for total body burdens (IOM, 2009). Once in the body, dioxins can be found in most tissues, but in general, studies suggest that dioxins are primarily distributed in the liver and adipose tissues (ATSDR, 1998). Distribution and elimination of dioxins can differ according to such factors as tissue type, magnitude of exposure, and time since first exposure. In studies of TCDD concentration in the body, the concentration of cytochrome P450 1A2 (CYP1A2) in the liver was found to increase with increased TCDD concentration. Binding of the two is believed to result in the sequestration of TCDD in the liver (Dragin et al., 2006; Poland et al., 1989; IOM, 2009). Distribution of TCDD has also been shown to be dependent on age (Pegram et al., 1995) and adipose tissue mass (Aylward et al., 2005; Emond et al., 2006).

Metabolism of dioxins in the body has thus far not been well characterized. Studies of TCDD have suggested that its breakdown occurs slowly, and there is some evidence from animal studies that the breakdown products are less toxic than TCDD. Dioxins and their metabolites are primarily eliminated in feces (and in urine, to a lesser extent). They may also leave the body in breast milk from nursing mothers (ATSDR, 1998). There is also evidence that suggests that the elimination of TCDD is dose-dependent, with more TCDD detected in feces at higher doses (Diliberto et al., 2001). This may be due to increased expression of metabolizing enzymes at higher doses (IOM, 2009). In humans, such factors as gender, age, body mass index (BMI), and size of dose can affect the average time required to eliminate

one-half of the TCDD from the body (called the “half-life” of TCDD). Estimates of the half-life have ranged from as low as 0.4 years to as high as 10 years (ATSDR, 1998; IOM, 2009).

4. Mechanism of action: The aryl hydrocarbon receptor pathway

The biochemical effects of dioxins are mediated by the activation of the AhR, which belongs to a family of ligand-activated basic helix-loop-helix (bHLH) transcription factors (Beischlag et al., 2008; IOM, 2009). The bHLH proteins are characterized by their structure, which consists of a sequence of basic amino acids and two alpha helices joined into a loop. They are involved in the transcription of genetic information from deoxyribonucleic acid (DNA) to ribonucleic acid (RNA). They are dimeric and form functional heterodimers with other members of the family. In general, the larger of the two alpha helices is involved in binding to DNA at a specific sequence. Binding of the heterodimeric complex to DNA seems to recruit transcriptional machinery that increases the production rate of messenger RNA (mRNA) for the genes regulated by the complex (which ultimately leads to a corresponding increase in the associated protein) (IOM, 2009).

Different regions of the AhR, from the amino-terminus to the carboxyl-terminus of the protein, perform different functions. The AhR is a member of the PER-ARNT-SIM (PAS) bHLH subfamily (IOM, 2009; White and Birnbaum, 2010). All members of this subfamily share the bHLH motif, located at the amino-terminus (Jones, 2004). The basic region of the protein participates in the binding of AhR heterodimers to DNA. The HLH region aids the heterodimerization of the AhR and the AhR nuclear translocator (ARNT), which is discussed in further detail below. The PAS domain consists of a sequence of 200–350 amino acids that are highly related to domains originally found in “period” (Per) and “single minded” (Sim) genes of the Drosophila melanogaster and in the ARNT (IOM, 2009). The AhR has two PAS domains—PAS-A and PAS-B—that participate in interactions with other proteins (e.g., other PAS-carrying proteins, chaperones, other transcription factors). The PAS-B domain contains the ligand

binding site (Ema et al., 1992; IOM, 2009). One other important domain, with a structure rich in glutamine, is found in the carboxyl-terminus. Its role is tied to coregulator recruitment and transactivation (IOM, 2009; Kumar et al., 2001).

When not bound to a ligand, the AhR resides in the cell cytoplasm, where it interacts with several chaperone proteins. These include two heat shock protein 90 (Hsp90) molecular chaperones, one prostaglandin E synthase 3 (p23) protein, and one immunophilin-like protein hepatitis B virus X-associated protein 2 (XAP2). After diffusion into the cell, dioxins bind the AhR, which then releases one molecule of Hsp90 as well as both p23 and XAP2. The AhR-dioxin complex next translocates into the nucleus, where it heterodimerizes with the ARNT at their respective PAS domains. Only after heterodimerization and the release of the remaining molecule of Hsp90 can transcription occur. Transcription begins when the AhR-dioxin-ARNT complex binds to DNA at dioxin response elements (DREs) found in the promoter region of target genes. The ability of the complex to initiative transcription may be altered by coactivators (e.g., thyroid hormone receptor/retinoblastoma protein-interacting protein 230) or corepressors (e.g., estrogen receptor alpha) (White and Birnbaum, 2010). A visual representation of AhR activation is displayed in Figure 1.

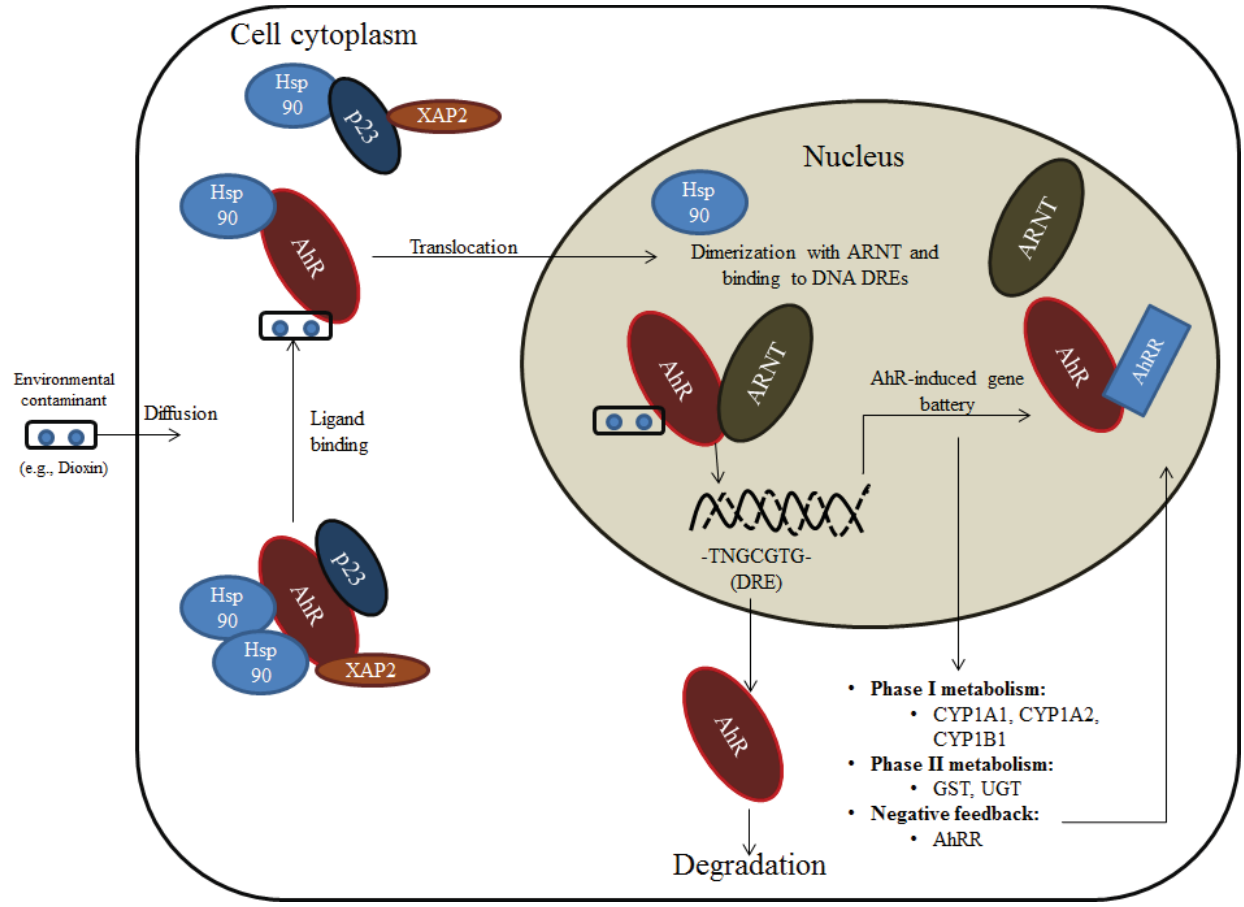

Figure 1. Simple diagram of AhR signal transduction.

Dioxins exert their toxicity by persistently activating the AhR and thus preventing it from performing its normal homeostatic functions. The AhR appears to be necessary but not sufficient for most dioxin-associated toxicity (White and Birnbaum, 2010). It may play a role in cell-cycle control and tumor suppression in the stomach, colon, prostate, and hematopoietic tissues (Marlowe and Puga, 2005; Singh et al., 2008). Thus, disruption of normal functioning could potentially lead to adverse outcomes. Persistent activation of the AhR has been shown to affect processes related to cell proliferation, adipose differentiation, apoptosis, tumor promotion, and immune and endocrine function (Abel and Haarmann-Stemmann, 2010). It may also lead to increased competition for

heterodimerization with the ARNT, displacing other potentially important signaling partners (White and Birnbaum, 2010). The AhR repressor (AhRR) is a negative regulator of AhR activity. The AhR action induces gene and protein expression of the AhRR, indicating that AhR and AhRR form a regulatory feedback loop (Hahn et al., 2009; Kawajiri and Fukii-Kuriyama, 2007).

The AhR-dioxin-ARNT complex stimulates the transcription of genes other than the AhRR as well, especially those coding for xenobiotic metabolizing enzymes. These include cytochrome P450 enzymes (CYPs), aryl hydrocarbon hydroxylase, and ethoxyresorufin o-deethylase (Nagao et al., 1990; Safe et al., 1989). The structural similarities of PCDDs, PCDFs, and dioxin-like PCBs suggest that they activate the AhR and exert their toxic effects via the same mechanism.

B. **Dioxin Exposure and Non-Hodgkin's Lymphoma**

In 1997, the International Agency for Research on Cancer classified TCDD as carcinogenic to humans. This taxonomy was based on limited evidence of carcinogenicity in humans, sufficient evidence of carcinogenicity in rodents, and strong evidence of a common mechanism in both humans and animals—initial binding of TCDD to the AhR, which leads to alterations in gene expression, cell replication, and apoptosis (IARC, 1997). A reassessment conducted in 2009 reaffirmed TCDD's carcinogenicity in humans, based on more recent findings of increased risk of all cancers combined (Baan et al., 2009). The etiology of non-Hodgkin's lymphoma (NHL) is not well understood, as is the case with many malignancies. However, studies of individuals exposed to chlorophenols, phenoxy acids, and their dioxin contaminants, coupled with our current understanding of dioxin's mechanism of action, suggest an association between dioxin exposure and NHL. What follows includes a description of the disease, a discussion of issues encountered in the design of epidemiological studies of cancer, and a relatively brief overview of relevant findings from the literature with regard to the relationship between dioxin exposure and NHL.

1. **Non-Hodgkin's lymphoma**

Non-Hodgkin's lymphoma (NHL) refers to a rather diverse group of malignant neoplasms of the lymphoid tissues, with varying clinicopathological and biological characteristics. These neoplasms derive from the clonal expansion of B-cells, T-cells, natural killer cells, or from the precursors of these cells (Müller et al., 2005). The heterogeneity in this group of malignancies, which all originate in the lymphocytes, prompted the creation of several lymphoma classification systems. Currently, a categorization adopted by the WHO reigns. This system, adapted from the Revised European-American Classification of Lymphoid Neoplasms, classifies NHL according to cell-type affected as well as phenotypic, molecular, and cytogenetic characteristics (Ekström-Smedby, 2006; Fisher and Fisher, 2004). Diffuse large B-cell lymphoma, follicular lymphoma, Burkitt lymphoma, and mantle-cell lymphoma are among those classified as B-cell NHLs. Malignancies such as anaplastic large-cell lymphoma, mycosis fungoides, and precursor T-lymphoblastic lymphoma (IOM, 2009) are classified as T-cell NHLs.

Non-Hodgkin's Lymphoma ranks as the fifth-most common cancer in the United States, with approximately 65,000 people diagnosed in 2010, and the sixth-most common cause of cancer mortality. It accounts for 4% of all new cancer cases in the United States, and incidence has doubled since the 1970s, having risen by about 1%–4% per year over the past century (Howlader, 2015). Most of this increase is seen in high-grade, potentially curable subtypes (Müller et al., 2005).

Non-Hodgkin's Lymphoma occurs most frequently in middle-aged and older adults (50 years of age or older), a demographic that continues to grow with the general population due to ever-increasing life expectancy. The most common subtypes of NHL are diffuse B-cell lymphoma (approximately 30%) and follicular lymphoma (approximately 20%). All others individually make up less than 10% of the total. Men are at increased risk of developing many of the subtypes (mantle-cell lymphoma, most notably), though follicular lymphoma more frequently occurs among women (Ekström-Smedby, 2006). White individuals seem to be disproportionately affected by NHL when compared with Black individuals (Fisher

and Fisher, 2004). Though therapeutic advancements have improved survival for some NHL subtypes (such as diffuse large B-cell lymphoma), NHL remains an emerging threat globally.

2. Previous findings

The etiology of NHL is not yet well understood, as is the case with many malignancies. Epidemiological studies suggest the possibility of a causal role for exposure to chlorophenols, phenoxy acids, and their dioxin contaminants in NHL development. Measures of association estimated in these studies are not always strong or statistically significant, but researchers have tended to find higher than expected rates of NHL in those occupationally exposed to this class of chemicals. The risk for NHL has been significantly linked to exposures experienced by phenoxy herbicide producers in the Netherlands (Boers et al., 2012) and Germany (Becher et al., 1996). Nonstatistically significant increases in NHL mortality were observed in several other cohorts of chemical workers (Zober et al., 1990; Kogevinas et al., 1997; Steenland et al., 1999; Bodner et al., 2003). Pesticide applicators have been shown to have a significantly increased risk of NHL development (Hardell et al., 1981; Olsson and Brandt, 1988), as have Kansas farmers (Hoar et al., 1986). Studies of farmers from other areas of the United States have demonstrated nonstatistically significant increases in NHL risk (Zahm et al., 1990; Cantor et al., 1992; Hansen et al., 2007), as have studies of paper and pulp mill workers (Robinson et al., 1986; Jappinen and Pukkala, 1991; Hennenberger et al., 1989). Additionally, studies of environmentally exposed residents in Seveso, Italy—an area contaminated by an industrial accident in the summer of 1976 that exposed thousands of people to substantial levels of TCDD—suggest an increased risk of NHL (Bertazzi et al., 2001; Consonni et al., 2008). Risk was also found to increase significantly with increasing serum organochlorine levels in residents living near waste incinerators (Viel et al., 2000). There is further evidence of an association between NHL and exposure to dioxin-like and non-dioxin-like PCBs (Engel et al., 2007; Spinelli, 2007; Freeman and Kohles, 2012).

C. **Dioxin Exposure, Translocations, and Non-Hodgkin's Lymphoma**

The t(14;18) translocation is widely recognized as the hallmark genetic event in the development of follicular lymphoma (de Jong, 2005). The t(14;18) arises when the BCL-2 (B-Cell Leukemia/Lymphoma 2) gene on chromosome 18q21 is translocated to a position near the immunoglobulin heavy chain (IgH) locus at chromosome 14q32—this transfer between chromosomes occurs in a reciprocal manner (Tsujimoto et al., 1985). When this occurs, the IgH gene promoter stimulates expression of the anti-apoptotic BCL-2 gene, resulting in the excess production of BCL-2 protein (McDonnell and Korsmeyer, 1991). The majority of t(14;18) translocations (approximately 70%) involve what is called the major breakpoint region, which is located in the untranslated region 3' of the BCL-2 gene's last exon (Biagi and Seymour, 2002). The BCL-2 protein, located in the inner mitochondrial membrane, prolongs the survival of certain cells by preventing apoptosis (McDonnell and Korsmeyer, 1991). The overexpression of a structurally intact and functional BCL-2 anti-apoptotic protein leads to accumulation of inappropriately rescued long-lived B cells. This survival advantage likely predisposes cells with the BCL-2 mutation to further DNA damage (Biagi and Seymour, 2002). The process of malignant transformation of a normal B-cell into a malignant cell is a multistep process. The t(14;18) translocation appears to be a necessary, but not sufficient, step for transformation of the cell to a malignant state (Limpens et al., 1991; Sehn, 2006).

The clinical significance of the t(14;18) translocations found in healthy subjects is unresolved. However, those with a large number of circulating cells with the t(14;18) would be expected to have a higher risk of developing lymphoma compared to those with fewer such cells, given that the t(14;18)-containing cells would be more likely to sustain the necessary additional genetic changes (Fuscoe et al, 1996). Additionally, clonal expansion of circulating t(14;18)-positive cells may further exacerbate this risk. This expansion could potentially be stimulated by carcinogenic exposure and lead to further molecular and cytogenetic adverse events (Hoglund et al, 2004).

Several studies of risk factors for t(14;18) prevalence and frequency in healthy individuals have been conducted, though findings have been mixed. Demographic factors found to be associated with t(14;18) prevalence include race, with Asians having a lower risk (Yasukawa et al., 2001; Schüler et al., 2003); and age, with older individuals at increased risk (Schüler et al., 2003; Nambiar and Raghavan, 2010). Fair hair and light eye color have also found to be associated with an increase in t(14;18) prevalence in healthy individuals (Baccarelli et al., 2006). Higher frequency, or increased expansion, of the chromosomal translocation has been found to be positively associated with age (Schüler et al., 2003; Hirt et al., 2007) and smoking (Baccarelli et al., 2006; Hirt et al., 2007). Agricultural pesticide use seemed to act as a risk factor for increased t(14;18) frequency in the blood (Schroeder et al., 2001; Roulland et al., 2004; Agopian, 2009), while another research group demonstrated a dose-response relationship with plasma TCDD levels in a sample of Seveso, Italy residents (Baccarelli et al., 2006). Hepatitis-C virus infection (Zignego et al., 2002) and exposure to ultraviolet B from sunlight (Bentham et al., 1999) were also found to be associated with increased t(14;18) frequency.

Studies of relationships between various risk factors and t(14;18)-positive lymphomas highlight other potential influences on t(14;18) prevalence and clonal expansion. Factors that were found to increase the risk of t(14;18)-positive NHL include pesticide use (Chiu et al., 2006), family history of hematopoietic cancer (Chiu et al., 2007), taller height (Chang et al., 2011), more lifetime surgeries (Chang et al., 2011), and PCB-180 exposure (Chang et al., 2011). Associations (both positive and negative) have also been demonstrated for dietary factors, such as milk, dietary nitrite, and coffee consumption (Chiu et al., 2008; Chiu et al., 2011). It should be noted that risk factors did not typically coincide from study to study, whether investigating healthy individuals or t(14;18)-positive NHL cases. For instance, Chiu et al. found that those who smoke more were more likely to develop the t(14;18)-negative, NHL subtype. Other studies found no correlation between age or gender and t(14;18) prevalence or frequency (Schmitt et al., 2006).

Though exposure to pesticides and TCDD have been found to be associated with t(14;18) translocations, study of the relationship between specific PCB congeners and t(14;18) prevalence and frequency in healthy individuals is lacking. However, researchers have begun to investigate the role of PCBs in BCL-2 gene expression. In an attempt to characterize how PCBs may modify the toxicity of polycyclic aromatic hydrocarbons (PAHs), one research team found that both NDL-PCB 101 and DL-PCB 126 prevented benzo[a]pyrene (BaP)-induced apoptosis in HepG2 cells; BCL-2 levels were found to be higher than would be expected in the response to BaP alone (Al-Anati et al., 2010). Another group differentiated acute and chronic effects of PCB exposure. Models of acute exposure to PCB 77 (a DL-PCB) and PCB 153 (an NDL-PCB) using HepG2 cells suggested heightened apoptotic activity via distinct molecular pathways. The PCB 153 was hypothesized to influence apoptosis through a mitochondrial pathway, whereas PCB 77 was posited to act through an AhR-mediated nuclear pathway (De et al., 2010). Interestingly, the chronic exposure of HepG2 cells to PCB 153 resulted in an up-regulation of BCL-2 expression (Ghosh et al., 2007).

D. Interaction of Dioxins and Non-Dioxin-Like Polychlorinated Biphenyls

1. Dioxin exposure and the aryl hydrocarbon receptor pathway

As discussed previously, the adverse health effects associated with dioxin exposure are mediated through ligand-binding to and persistent activation of the AhR, a cytosolic transcription factor (Safe, 2001; Puga et al., 2009). Persistent activation of the AhR can prevent it from functioning in the maintenance of homeostasis, with evidence suggesting that dioxin toxicity represents a hyperactivation of AhR functioning (Cèspedes et al., 2010). Briefly, the dioxin binds to the AhR (different congeners bind with different affinities), and this complex then translocates into the cell nucleus, where it dimerizes with the ARNT protein and subsequently binds toDREs. Several detoxification genes are transcriptionally induced as a consequence of AhR activation, including those coding for Phase I xenobiotic-metabolizing

cytochrome P450 enzymes (e.g., CYP1A1, CYP1A2, CYP1B1, CYP2S1), Phase II enzymes (e.g., UDP-glucuronosyl -transferase UGT1A6, NAD(P)H-dependent quinine oxydoreductase-1 NQO1), aldehyde dehydrogenases (e.g., ALDH3A1), and several glutathione-S-transferases (Puga et al., 2009). The AhR activity further activates genes that encode the AhRR protein, which regulates expression of the AhR itself, as well as other proteins involved in cell growth, migration, and death (Hahn et al., 2009). Further, DNA microarray analyses in HepG2 cells have revealed 310 genes upregulated or downregulated by TCDD treatment (Puga et al., 2000). Thus, the number of genes expressed as a result of AhR activation is much larger than originally thought and is not limited to genes encoding components of drug metabolism (Abel and Haarmann-Stemmann, 2010). It has also been suggested that different congeners may selectively modulate the AhR, as seen in a study showing different gene expression patterns in mouse Hepa1c1c7 hepatoma cells and C57BL/6 mouse liver samples treated with TCDD versus PCB 126 (Nault et al., 2013).

In-vivo studies of the expression of genes in the AhR pathway as it relates to dioxin exposure are currently lacking. An analysis of uncultured bovine peripheral blood lymphocytes demonstrated a detectable level of AhR, ARNT, and CYP1B1 expression, whereas CYP1A1 was expressed only when cultured with a mitogen. The researchers also found that CYP1B1 seemed to be significantly induced by dioxin exposure (Girolami et al., 2013). Similar findings were obtained in human uncultured lymphocytes sampled from residents exposed to dioxins in Seveso, Italy (Landi et al., 2003; Baccarelli et al., 2004).

2. **Aryl hydrocarbon receptor pathway antagonism**

Risk assessment for dioxin toxicity commonly incorporates the TEQ methodology, described previously. This approach is based on a relative toxic potency ranking scheme, where each dioxin congener is assigned a TEF. The TEFs are approximated using data collected from in-vitro and animal studies, assuming these are predictive of toxicity in humans. This may be a relatively valid

assumption, but there have been studies that advocate caution. One study found that TCDD, 3,3,4,4,5-pentachlorobiphenyl (PCB 126), and Aroclor 1254 were 100–1000 times more potent in rat and monkey cells than in human cells, as measured by CYP1A1 and CYP1A2 gene expression in species-specific hepatocytes (Silkworth et al., 2005). The TEQ estimate also assumes that the toxicokinetics and the toxicodynamics of all congeners are similar and that the dose-response curves of the congeners are similarly shaped. It then follows that total exposure is assumed to be additive (Van den Berg et al., 2006). While the empirical data on TCDD and some of the other dioxin-like congeners are consistent with dose additivity, the assumptions of the TEQ methodology are still clouded with uncertainty (EPA, 2010). The experimental conditions used to assess relative contributions of congeners are also not consistent with the real-world exposures seen in human populations. People are typically exposed to assorted mixtures of contaminants that may interact to affect toxic response. Further, positive correlations between dioxin and NDL-PCB exposure may lead to erroneous interpretation of the causal relationship between exposure and response (Longnecker et al., 2000).

Some data suggest that combined exposures to some dioxin-like congeners may have antagonistic (rather than additive) effects, and that these effects may be species-specific (EPA 2010). There is also evidence that NDL-PCBs may exhibit antagonistic activity for some responses (Safe, 1998). Several NDL-PCBs have been identified in experimental studies to have antagonistic effects on dioxin-mediated response (Bannister et al., 1987; Haake et al., 1987; Biegel et al., 1989; Davis and Safe, 1989). It has been suggested that under conditions where TCDD elicits a submaximal AhR-mediated response, the presence of a second full agonist will enhance this response. On the other hand, under conditions where TCDD elicits a maximal response, a partial agonist or antagonist compound will inhibit the response through unproductive AhR binding. It would seem that various planar, nonpolar molecules can bind the AhR but differ in their ability to trigger conversion to the DNA-binding form. While coplanar PCBs (e.g., PCB 77, PCB 126) and mono-ortho PCBs (e.g., PCB 156) have been shown to act as agonists of

the TCDD response, as measured by increased CYP1A1 gene transcription and protein levels, nonplanar PCBs (e.g., PCB 153) have been observed to be antagonists (Chen and Bunce, 2004). Another research team found that AhR-mediated expression of uridine glucuronyl transferase isozyme UGT1A6 was induced by DL-PCBs (compared to NDL-PCBs) only (Hamers et al., 2011). A recent analysis of peripheral blood mononuclear cells (PBMCs) revealed that the gene expression response induced by DL-PCB 126 differed from that of several NDL-PCBs. The study team concluded that exposure to PCB 126 activated the AhR signaling pathway, and exposure to NDL-PCBs did not seem to induce nuclear receptor pathways in blood cells (Wens et al., 2011). Overall, it seems that exposure to NDL-PCBs may modify the body's AhR-mediated response to dioxin-like compounds, and this modification may be characterized through gene expression analysis.

E. **Dioxin Exposure and Chloracne**

Exposure to TCDD and other diaromatic organochlorine chemicals can cause the onset of a skin condition known as chloracne. In fact, the development of chloracne represents a well-validated indicator of exposure to high doses of TCDD and other dioxin congeners—it is one of the few clinical findings in humans consistently associated with such an exposure (IOM, 2009). As such, chloracne has been the subject of numerous studies of cohorts exposed to dioxins.

Clinically, chloracne cases present with follicular hyperkeratosis (comedones) that may or may not occur with further inflammatory nodules and noninflammatory straw-colored cysts. Any part of the body can be affected, though chloracne is usually observed on the face, neck, and genitals. It can develop relatively soon after exposure to chloracnegens—usually within a few weeks—and may clear up in as short as several months after exposure ceases or as long as 30+ years after initial onset (ATSDR, 1998; Ju et al., 2012). Histopathology includes miniature-to-absent sebaceous glands, hyperplasia of the epidermis, and hyperpigmentation of the stratum corneum (Ju et al., 2012).

The German doctor S. von Bettman first described the condition in 1897, with Dr. Karl Herxheimer coining the term “chloracne” two years later (Ju et al., 2012). Since then, there have been numerous descriptions of this skin disease in the literature, resulting from occupational exposure to dioxins (Beck et al., 1989; Bond et al., 1987; Cook et al., 1980; Oliver, 1975; Suskind and Hertzberg, 1984; Zober et al., 1990). Chloracne has also developed in workers and residents as a result of industrial accidents involving release of dioxins. A rash of severe chloracne cases were documented following an explosion that occurred at a trichlorophenol plant in Nitro, West Virginia in 1949 (ATSDR, 1998). Similar events that spawned chloracne cases in workers due to industrial poisoning occurred in Czechoslovakia (Jirásek et al., 1976) and at an English chemical plant in Derbyshire (May 1973), among others. There have also been several studies of skin lesions in residents of Seveso, Italy—the site of a 1976 industrial accident—linking dioxin exposures and chloracne (Baccarelli et al., 2005; Caputo et al., 1988; Mocarelli et al., 1991). Ingestion of PCB-contaminated oil resulted in massive intoxication on two occasions: “Yusho” in Japan in 1968 and “Yu-Cheng” in Taiwan in 1979. These exposures resulted in increased risk of chloracne development (Guo et al., 1999; Yoshimura, 2003). Though no chloracne was reported among Times Beach, Missouri, residents exposed to TCDD-contaminated oil sprayed onto roads or among Vietnam War veterans involved in Operation Ranch Hand (defoliation project that exposed involved personnel to dioxin-contaminated herbicides), it is believed that exposure to dioxin in these scenarios was not as severe (ATSDR, 1998).

As stated previously, the AhR signaling pathway seems to mediate most of the effects of TCDD and other dioxin-like congeners. It follows that there are several studies that implicate the AhR pathway in chloracne pathogenesis (Tauchi et al., 2005; Tang et al., 2008; Ju et al., 2011). Expression of the AhR has been demonstrated in human keratinocytes, skin fibroblasts, sebaceous glands, and immortalized SZ95 sebocytes (Bock and Köhle, 2009; Ju et al., 2011). One study also saw the induction of B lymphocyte maturation protein 1 (Blimp1) in sebaceous glands upon treatment with AhR ligands (Ikuta

et al., 2010). In another study that compared twelve dioxin-exposed chloracne cases to twelve healthy controls, the investigators reported that p-epidermal growth factor receptor, p-mitogen-activated protein kinase, and CK17 were activated or upregulated to a greater extent in the case group (Liu et al., 2011). In 2004, Ukrainian president Victor Yushchenko consumed a meal poisoned with TCDD. Gene expression analysis of his skin cells revealed the induction of CYP1A1, CYP1A2, CYP1B1, and the AhRR. The AhR itself, its Hsp90 and prostaglandin E synthase 3 (p23), and the ARNT genes were not differentially expressed (Saurat et al., 2012).

Though development of chloracne is indicative of very high exposure to chloracnegens, very high exposure to dioxins does not guarantee chloracne development (Beck et al., 1989). This finding is supported by studies of residents with documented exposure to TCDD after the Seveso, Italy, accident (Baccarelli et al., 2005). Attempts have been made to quantify the blood levels necessary to develop chloracne (Coenraads et al., 1999; Neuberger et al., 1991), though the dissimilarities in study findings suggest that there may not necessarily be a correlation between serum dioxin concentration and the occurrence of chloracne (IOM, 2009). Case studies also indicate individual variations in disease severity (Geusau et al., 2001), suggesting that there may be factors other than exposure level that predict how serious symptoms will be. Further study into the mechanisms by which some individuals with high dioxin exposure are protected from chloracne development would greatly contribute to the understanding of how dioxins exert their toxicity.

F. **Smoking and the Aryl Hydrocarbon Receptor Pathway**

Cigarette smoke contains thousands of chemicals, some of which are known agonists of the AhR. These include dioxins (PCDDs, PCDFs, and coplanar PCBs) and polyaromatic hydrocarbons (e.g., benzo[a]pyrene and naphthalene) (Kitamura and Kasai, 2007). The total amount of dioxins in cigarette smoke has been estimated at 0.4 to 2.4 picograms of total TEQ (pg-TEQ) per cigarette; for a 60 kg

individual who smokes 20 cigarettes per day, this would translate to 0.14 to 0.96 pg-TEQ per kg body weight per day, which is below the WHO proposed total daily intake level of 1–4 pg-TEQ per kg body weight per day (Aoyama et al., 2003; Kitamura and Kasai, 2007).

Available evidence suggests that smoking may exert its toxicity at least partially via activation of the AhR pathway. A quantitative assessment of the effect of cigarette smoke extract on AhR stimulation found that the DRE activating potential of CSE was comparable to that of dioxins both in vitro and in vivo. Further, mice exposed to cigarette smoke in vivo showed increased AhR activation that persisted for several days without additional exposure, which may suggest that habitual smoking could cause sustained AhR activation in humans (Kasai et al., 2006).

The wide variety of biologically active chemicals found in cigarette smoke have the potential to modify other effects of interest, encouraging the assessment of interactions between smoking habits and other chemical exposures. Furthermore, given that constituents of cigarette smoke can activate the AhR, any study of the toxicity of dioxin exposure must account for the potential confounding effects of smoking habits.

II. METHODS

A. Study Design

The data used in the studies described herein were obtained thanks to the painstaking effort and dedication of a large interdisciplinary, multinational research team. Study protocols were initially developed by Dr. Irina Dardynskaia, Dr. Daniel Hryhorczuk, and others with the goal of attaining a deeper understanding of epidemiological and toxicological aspects of dioxin toxicity in humans. The general data collection methods, along with results from preliminary analyses of the data, are described in this section. Further detail, when relevant, is provided in the Methods sections of each of the three individual study manuscripts contained within this document.

1. Subjects

In 2009, 323 former workers with historical occupational exposure to dioxins while engaged in the manufacture of chlorinated phenols and their phenoxy-acid esters in an Eastern European chemical plant were recruited. For comparison, 150 individuals from the general population of a city located approximately 250 kilometers from the chemical plant with similar demographics were additionally enrolled. Factory workers and subjects in the population-based sample were frequency-matched on age, gender, ethnicity, and employment status. Informed consent was obtained from all participants. Study protocols were approved by the Centers for Disease Prevention and Control (CDC) National Institute for Occupational Safety and Health (NIOSH) and the University of Illinois at Chicago (UIC) Institutional Review Boards.

2. **Occupational exposures**

The plant from which workers were sampled was established in the 1940s and produced various chlorinated chemicals, ranging from household bleach to pesticides. The plant employed approximately 4,000 people and was closed in the early 2000s.

During its operation, the chemical plant accumulated about 530,000 m^3 of waste sludge with a dioxin content of 7–21 parts per billion (TEQ). In 1990, the dioxin gas emissions discharged by toxic waste incinerators at the plant was about 900g (total PCDDs/PCDFs). As a result of these environmental releases, the surrounding area is highly contaminated with dioxins.

The manufacture of lower chlorinated phenols at the plant began in 1960. Trichlorophenol (TCP) and its copper salt (TCP-Cu) were produced in three locations. Between 1961 and 1974, these chemicals were produced in one building of one department of the factory. The annual production capacity during this period was 120 tons of TCP and 35 tons of TCP-Cu. From 1975 through 1987, TCP and TCP-Cu were produced in two different buildings of the same department with a production capacity of TCP at 1,000 tons a year and of TCP-Cu at 100 tons a year. In 1987, the manufacturing of TCP and TCP-Cu was discontinued. The total production capacity of the plant has been estimated at 9,223 tons of TCP and 7,861 tons of TCP-Cu. Samples of technical 2,4,5-TCP contained up to 0.65 mg/kg of 2,3,7,8-TCDD.

The experimental production of the N-butyl esters of 2,4,5-T began in November 1964 in another department of the facility. The industrial manufacturing of this product began in May 1965 and was discontinued in 1968. The production capacity of 2,4,5-T was approximately 50–60 tons per month. A series of accidents in this department resulted in the contamination of the plant and surrounding territory along with excessive occupational exposure levels. During one of these accidents approximately 8 tons of 2,4,5-T were spilled on the floor, which subsequently traveled into the disposal system. Industrial hygiene controls were not adequate and worker overexposures occurred on numerous occasions (source: personal communication with plant workers).

The experimental manufacturing of esters of 2,4-D at the plant started in the early 1960s in another department of the facility. From 1963 through 1989, esters of 2,4-D (filtration scheme) were produced in a separate department. From 1986, the production of 2,4-D (extraction scheme) began in yet another department. In 1990, the plant began production of the sodium salt of the monochloroacetic acid of 2,4-D using modern technology. Metaxone (2-Methyl-4-chlorophenoxyacetic acid) was produced at the plant in one department from 1966 through 1989.

3. **Sampling and data collection**

Plant personnel records, as well as medical and occupational histories, were obtained. Qualified professionals performed physical examinations and collected blood samples for serum dioxin and biomarker studies. The subset of medical and occupational history data that were related to study hypotheses were entered into a database by the UIC Data Management Center in Kyiv, Ukraine, and subsequently transferred to the UIC. Blood samples were transferred to partner laboratories at the CDC, Griefswald University in Germany, and the University of Milan in Italy for analysis. The UIC research team obtained analytical results for blood samples from 218 of the 323 former factory workers and all 150 population-sampled participants. The remaining 105 workers for whom blood sampling results were not obtained were known to be primarily involved in the manufacture of 2,4-D. This production process has been shown to be contaminated primarily by higher chlorinated PCDDs rather than by TCDD (ATSDR, 1998). The exposure profile of these individuals was systematically different from that of the other workers, and this difference was qualitatively characterized. As a result, their exclusion was not expected to affect the validity of our analyses, given the research questions posed. Thus, only workers for whom blood sampling data was available (218 workers and 150 population-sampled participants) were included in the studies described in this document.

Trained and certified phlebotomists acquired 88.5 ml of whole blood from each participant: 50 milliliters (mL) for blood dioxin determination, 14 mL for t(14;18) translocation, and 14.5 mL for biomarker studies. For the dioxin analysis, blood was collected according to the CDC Laboratory Procedure for PCDDs, PCDFs, and non-ortho, or coplanar, PCBs (cPCBs), using high-resolution gas chromatography/isotope-dilution high-resolution mass spectrometry (HRGC/ID-HRMS), Method 28. Serum samples were analyzed for seven PCDDs, 10 PCDFs, 4 cPCBs, and 38 ortho-substituted PCBs. Blood dioxin was processed and serum was frozen in a -80°C freezer prior to shipment. For t(14;18) translocation analysis, the standard operating procedure was provided by Griefswald University. Whole blood was collected in two EDTA-vacutainer tubes (each 7 mL) and the blood was poured into leucosep tubes prefilled with Ficol solution; phosphate-buffered saline (PBS) was added to buffer up to a total volume (Ficoll + blood + PBS) of 45 mL per tube. Mononuclear cells were separated by Ficoll Hypaque density gradient centrifugation at 1000 g for 10 minutes. The buffy coat containing the mononuclear cells was resuspended in 45 mL of PBS, centrifuged at 300 g for 5 minutes; the pellet was resuspended again in 45 mL of PBS. After centrifugation at 300 g for 5 minutes, the cell pellet was resuspended in 1 mL of PBS and the cell count was determined. After centrifugation, cell pellets (supernatant liquid removed) containing at least $1*10^7$ cells in 1.5 mL centrifuge tubes were stored in at least 2 aliquots at -80°C. For RNA expression studies, an additional 28.5 mL of blood were collected in the following tubes: three PAX tubes (2.5 mL of blood in each tube) for subsequent RNA extraction and one ethylenediaminetetraacetic acid (EDTA) vacutainer tube (7 mL of blood) for DNA extraction. Blood for biomarker studies was frozen in a -80°C freezer prior to shipment. The RNA was extracted from blood samples using the PAXgene blood RNA kit (Qiagen) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (PCR) was performed to detect the t(14;18) in PBMCs , as described previously by Dölken et al. (1998), using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). The DNA was obtained from roughly 140,000 cells, based on

spectrophotometric measurements. As a reference, PCR of wild-type K-ras was performed. The reporter dye FAM (6-carboxy-fluorescein) and the quencher dye TAMRA (6-carboxy-tetramethylrhodamine) were used to label probes at the 50-end and 30-end, respectively. The PCR mix was made up of 400 nM of forward and reverse primers, 200 nM of probe, the TaqMan Universal PCR Master Mix (PE Applied Biosystems), and 0.1 to 2.0 μg of DNA. This mixture was incubated for two minutes to allow cleavage by Uracil-N-Glycosylase (UNG), followed by activation of AmpliTaq Gold by incubation at 95°C for ten minutes. The denaturation step for each PCR cycle was set for fifteen seconds at 95°C, with the annealing/extension step of each cycle set for one minute at 61°C.

For detecting t(14;18), the standard operating procedure was carried out with five PCR reactions, using one μg of DNA, two positive controls, and two negative controls. Positive reference standards of t(14;18)-positive cell line Karpas 422 cloned fragments (200 or 700 copies per assay) were employed. Three PCR reactions using 0.1 μg of DNA were run to detect wild-type K-ras, with two positive and two negative controls. Aliquots of cloned wild-type K-ras (1,000 copies per assay) were used as positive reference standards for these PCRs. Additional DNA was examined when negative results were obtained for those groups of five aliquots that did not contain at least 350,000 cells.

The PCR technique described above has a sensitivity of one copy in 1–2 μg of DNA, or the ability to detect one t(14;18)-positive cell in 140,000–280,000 normal cells (Dölken et al. 1998).

For the gene expression analyses, a stratified random sample of 60 exposed subjects, based on tertiles of serum TCDD level, was selected from the population of 218 exposed subjects for whom RNA samples were archived. Also selected was a random sample of 30 unexposed subjects from the population of 150 unexposed participants, frequency-matched to the 60 exposed subjects on age, gender, ethnicity, and presence/absence of t(14;18) translocation. Matching on t(14;18)-positivity was performed to ensure adequate sample size in studies of the association between gene expression and t(14;18) frequency. Preliminary analysis of the data for the larger cohort did not support a significant

relationship between dioxin or PCB exposures and t(14;18)-positivity, leading us to conclude that matching on this factor would not greatly bias the results obtained for the gene expression subset. Qiagen prepared a customized array for 83 genes selected by the investigators. The criteria for selecting candidate genes were: (1) Key components of the AhR, human lymphoma, and apoptosis pathways; (2) >1.30-fold up-regulation or 0.65-fold down-regulation in previous studies of dioxin-exposed humans; or (3) >5-fold change in expression in previous studies of dioxin-exposed human cell lines. Gene expression was analyzed at the University of Milan using reverse transcription polymerase chain reaction (RT-PCR), a method used to amplify small amounts of DNA or RNA by several orders of magnitude. Messenger RNA (mRNA) is the result of DNA transcription, ready to be translated into functional protein. Thus, mRNA represents genes that will be expressed. Amplifying messenger RNA (mRNA) by RT-PCR in two samples allows one to compare gene expression between those samples. The relative amount of PCR product is measured by fluorescence, using SYBR chemistry. The RT-PCR procedure produces the cycles to threshold (Ct), or the number of PCR cycles at which the fluorescence generated within a reaction crosses the fluorescence threshold, for each gene in the PCR array. Theoretically, each PCR cycle should result in a doubling of cDNA.

The PAXgene Blood RNA Kit was used to isolate total RNA (Qiagen-PreAnalytix, Hombrechtikon, Switzerland). Complementary DNA (cDNA) was synthesized with an RT2 First Strand kit (SABiosciences, Frederick, Maryland, USA) and analyzed with specific RT2 Profiler PCR arrays by real-time PCR (RT-PCR) using Syber Green chemistry. The PCR array is a complete system for pathway-focused gene expression analysis with some components that guarantee high-quality, reproducible, and reliable gene expression data. The Profiler PCR array includes 89 RT2 quantitative PCR (qPCR) primer assays (including 5 housekeeping genes) and a proprietary control panel to investigate contamination with genomic DNA (gDNA), quality of the reverse transcription reaction, and real-time efficiency (QIAGEN Inc., Mississauga,

Canada). All RT-PCR runs were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, California, USA).

All data sets received at UIC were cleaned and merged into a single database, using SQL and SAS coding in SAS 9.3 (SAS Institute, Cary, North Carolina) to deterministically link information from various sources to each subject with a unique identification number. The SAS coding was also used to aggregate raw Ct RT-PCR values, calculate the mean of the Ct values for housekeeping genes, and normalize samples relative to the mean Ct of the housekeeping genes.

4. **Preliminary findings**

Demographics for the exposed factory workers, the presumably unexposed residents, and the excluded workers (those 105 workers without blood sampling results who were involved in the production of 2,4-D) are displayed in Table I. Frequency matching of the unexposed residents to the occupationally exposed workers on age, gender, ethnicity, and employment status was performed before exclusion of the 2,4-D production personnel. Exclusion of these workers affected the distribution of gender and ethnicity in the occupational and population-based cohorts. Thus, subsequent analyses were adjusted for these demographic factors to account for the distributional differences.

Figure 2 and Figure 3 show blood serum levels of each measured dioxin congener in the exposed factory worker cohort and in the unexposed group. These figures show that, despite the collection of blood samples years after working at the factory, the exposed sample of workers still has greatly elevated blood dioxin levels (TCDD in particular), on average, when compared with the unexposed group. More interestingly, both of the groups have similar DL-PCB levels—PCB 126 is highlighted, as it is the DL-PCB with the largest toxic equivalency factor (TEF=.1). Levels of NDL-PCBs are also comparable in both groups, with sizable variance. Selected dioxin and PCB congener levels are shown in Table II. Thus,

this study population affords a unique opportunity to study the effects of dioxin exposure, PCB exposure, and the interactions between them.

TABLE I

DEMOGRAPHIC CHARACTERISTICS OF STUDY COHORTS

	Exposed[a]		Unexposed[b]		Excluded[c]	
	#	%	#	%	#	%
Gender						
Male	125	57.3	76	50.7	51	48.6
Female	93	42.7	74	49.3	54	51.4
Ethnicity						
Caucasian	76	34.9	75	50.0	47	44.8
Asian	128	58.7	73	48.7	50	47.6
Other	14	6.4	2	1.3	8	7.6
Smoking Status						
Current	43	19.7	31	20.7	13	12.4
Former	41	18.8	29	19.3	24	22.9
Never	134	61.5	90	60.0	68	64.8
Age						
n	218		150		105	
Mean	67.5		66.8		67.7	
Std. Dev.	6.2		6.9		7.1	
Median	69.0		67.5		68.0	
Body Mass Index						
n	204		136		104	
Mean	28.1		28.9		30.1	
Std. Dev.	4.7		7.8		6.2	
Median	27.6		28.2		29.2	

[a] Includes 218 former chemical factory workers.

[b] Includes 150 population-based matched control subjects.

[c] Includes 105 factory workers for whom blood sample analysis were not received.

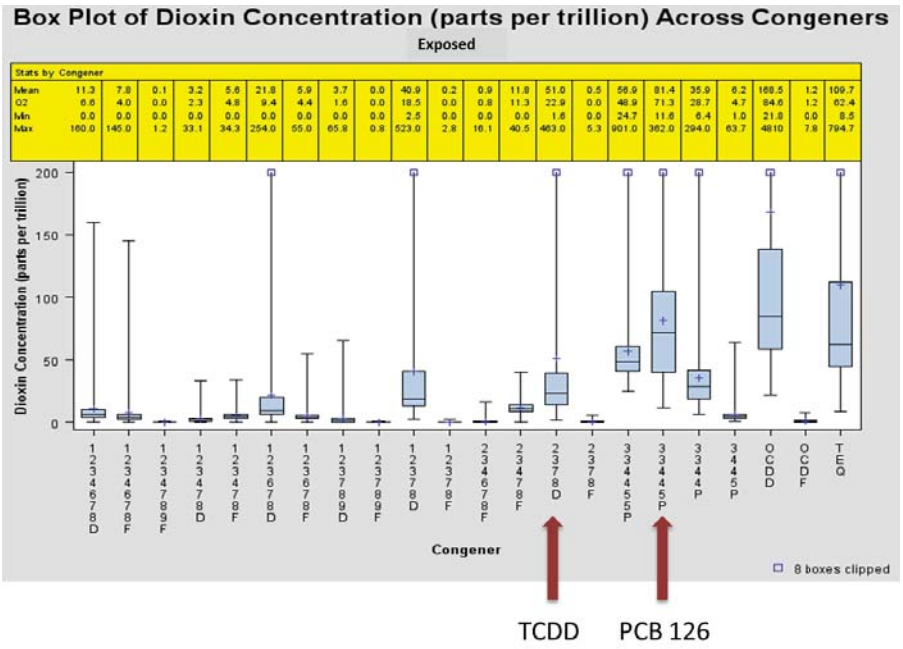

Figure 2. Blood serum dioxin levels measured in the factory worker cohort.

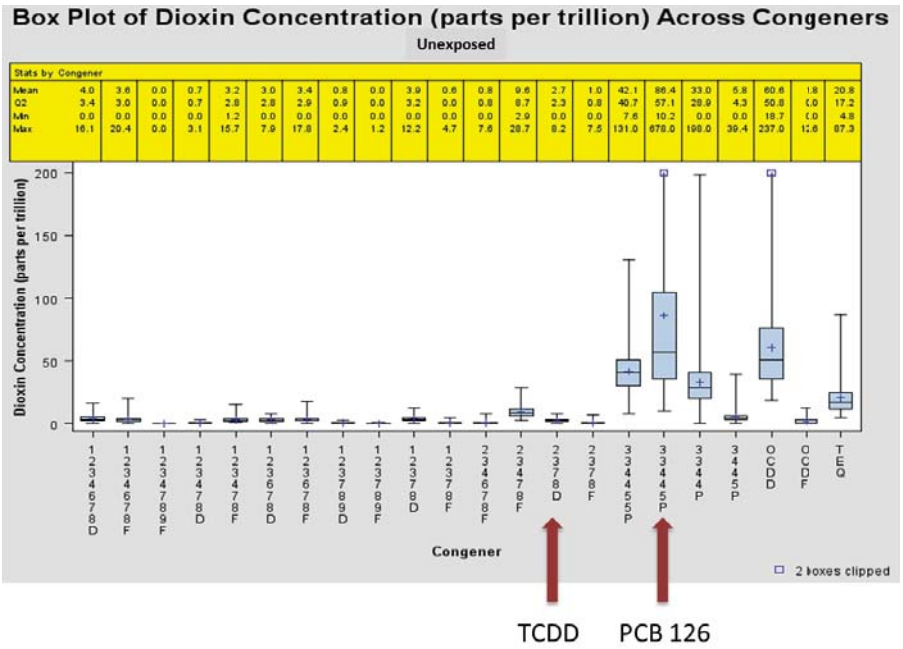

Figure 3. Blood serum dioxin levels measured in the population-selected control cohort.

TABLE II

BLOOD SERUM CONCENTRATIONS (PARTS PER TRILLION) OF SELECTED DIOXIN AND PCB CONGENERS IN THE FACTORY WORKER AND CONTROL COHORTS

Study Group	Variable	N	Mean	Std Dev	Median	Min	Max
Exposed Workers	2,3,7,8-TCDD	218	50.7	80.8	22.9	1.6	463.0
	PCB 126	218	81.6	57.0	71.3	11.6	362.0
	Total TEQ	218	109.3	124.8	62.4	8.5	794.7
	PCB 180	218	246.8	109.0	220.6	80.6	808.9
Population Controls	2,3,7,8-TCDD	150	2.7	1.7	2.3	0.0	8.2
	PCB 126	150	86.4	88.8	57.1	10.2	678.0
	Total TEQ	150	20.8	13.3	17.2	4.8	87.3
	PCB 180	150	149.6	92.1	133.1	24.9	916.7

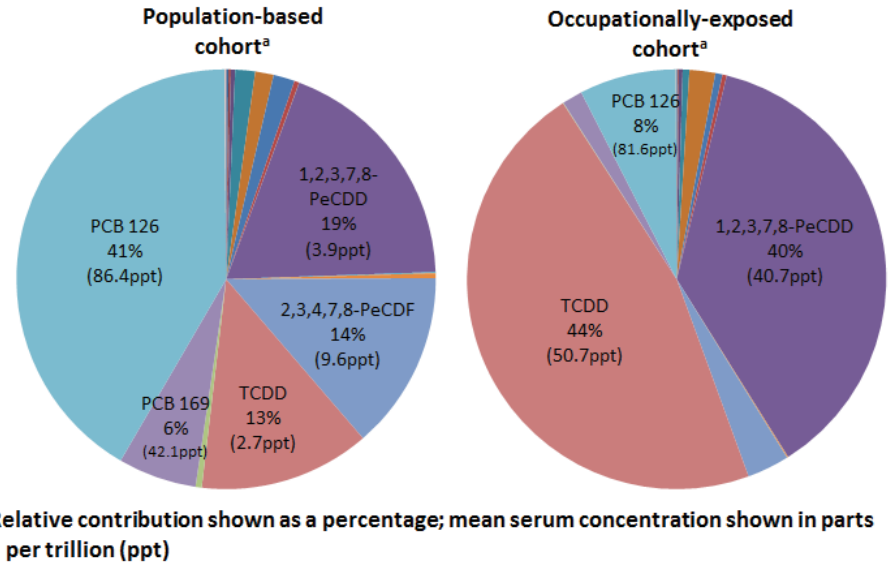

Figure 4. Relative contribution of each dioxin congener to the total toxic equivalency based on serum levels and toxic equivalency factors.

B. **Study Aims**

The overall goal of this research plan was to investigate the molecular toxicity of dioxins, specifically as it pertains to t(14;18) chromosomal translocations and AhR-mediated gene expression, in a cohort of chemical workers with past occupational exposure to dioxins and a group of population controls, frequency-matched on age, gender, ethnicity, and employment status. This was accomplished via three specific research questions and analysis methodologies, stemming from a common study design.

Described here are three analyses, using the data described above, aimed at improving our understanding of the health effects of dioxin exposure in humans. The outcome examined in the first study was a chromosomal aberration known as the t(14;18) translocation. As discussed, dioxin exposure has been linked to NHL in several epidemiological studies. The t(14;18) is found in a large proportion of some of the most common NHLs. A better understanding of the risk factors for t(14;18) prevalence and frequency may help to more effectively characterize those at risk of developing specific forms of NHL. In addition, an association between dioxin or PCB exposure and the chromosomal translocation may suggest a role for the t(14;18) as a biomarker of exposure to these chemicals. The first study involved an investigation of which demographic, behavioral, clinical, and/or environmental factors are associated with prevalence and/or frequency of the t(14;18) translocation in human lymphocytes. Given the high PCB blood serum levels in these populations (both the exposed and unexposed), the first study focused on the effect of DL-PCBs on t(14;18) translocations in the reference population sample.

The second study focused on elucidating the effects of complex chemical exposures on gene expression. More specifically, this study aimed to determine whether increased expression of genes in the AhR pathway due to dioxin exposure is antagonized by NDL-PCB exposure. While such relationships between dioxins and PCBs have been demonstrated in-vitro and in animal studies, no studies to date have tested this hypothesis in human subjects. The wide range of serum dioxin and PCB exposures in our

study group provide a unique opportunity to investigate the interplay between blood levels of different chemicals and gene expression. Because humans are typically exposed to a variety of chemicals (rather than just one at a time), it is essential that research be carried out to better understand how these chemicals interact in the human body.

The third study attempted to explain why some people with high serum TCDD levels do not develop chloracne—a condition commonly associated with heavy exposure to dioxins. Though several studies have estimated the blood levels of TCDD necessary to elicit chloracne development (as described previously), not all individuals with high blood levels develop noticeable symptoms. This study investigated whether any demographic/environmental factors, or whether differential gene expression, is associated with chloracne development. More specifically, the major hypotheses tested were: (1) that high concurrent exposures to NDL-PCBs antagonize chloracne development attributable to dioxin exposure, and (2) that expression of genes in the AhR pathway would be different in those who developed chloracne compared with those who did not. The answers to these research questions should provide some insight into the mechanisms involved in chloracne development in humans exposed to complex chemical mixtures. The results should also add to the growing body of knowledge related to the factors that affect dioxin toxicity at the individual level.

III. ENVIRONMENTAL EXPOSURE TO POLYCHLORINATED BIPHENYLS, SMOKING, AND THE RISK OF t(14;18) TRANSLOCATIONS IN HEALTHY INDIVIDUALS

A. Background

Dioxins are a class of chemical contaminants that have become ubiquitous in the environment, primarily as a result of industrial processes (ATSDR, 1998). Health effects that have been linked to dioxin exposure are numerous and include immune deficiency, reproductive/developmental abnormalities, nervous system disorders, endocrine disruption, skin conditions, and pulmonary disease (Schecter et al., 2006). In 1997, the International Agency for Research on Cancer classified TCDD as carcinogenic to humans (IARC, 1997). However, findings from studies of the relationship between dioxin exposure and specific cancer types have been mixed.

Non-Hodgkin's lymphoma refers to a rather diverse group of malignant neoplasms of the lymphoid tissues, with varying clinicopathological and biological characteristics. These neoplasms derive from the clonal expansion of B-cells, T-cells, natural killer cells, or from the precursors of these cells (Müller et al., 2005). The most common subtypes of NHL are diffuse B-cell lymphoma (about 30%) and follicular lymphoma (about 20%) (Ekström-Smedby, 2006).

The etiology of NHL has not been fully characterized in humans, as is the case with many malignancies. Epidemiological study of individuals exposed to chlorophenols, phenoxy acids, and their dioxin contaminants suggests an association between these exposures and NHL. However, the role of these chemicals in NHL development is debated, as measures of association estimated in these studies are not always strong or statistically significant. Risk of NHL has been significantly linked to exposures experienced by phenoxy herbicide producers (Boers et al., 2012; Becher et al., 1996), pesticide applicators (Hardell et al., 1981; Olsson and Brandt, 1988), and Kansas farmers (Hoar et al., 1986). Nonstatistically significant increases in NHL risk were observed in several other cohorts of chemical workers (Zober et al., 1990; Kogevinas et al., 1997; Steenland et al., 1999; Bodner et al., 2003), farmers

(Zahm et al., 1990; Cantor et al., 1992; Hansen et al., 2007), and paper and pulp mill workers (Robinson et al., 1986; Jappinen and Pukkala, 1991; Hennenberger et al., 1989). A larger than expected burden of NHL was noted in residents of Seveso, Italy, an area contaminated as a result of an industrial accident in the summer of 1976 that exposed thousands of people to substantial levels of TCDD (Bertazzi et al., 2001; Consonni et al., 2008). Similar risks have also been demonstrated in residents living near waste incinerators and in those environmentally exposed to PCBs (Viel et al., 2000; Engel et al., 2007; Spinelli, 2007; Freeman and Kohles, 2012).

The t(14;18) translocation is widely recognized as the hallmark genetic event in the development of follicular lymphoma (de Jong, 2005). The t(14;18) arises when the BCL-2 gene on chromosome 18q21 is translocated to a position near the IgH locus at chromosome 14q32—this transfer between chromosomes occurs in a reciprocal manner (Tsujimoto et al., 1985). When this occurs, the IgH gene promoter stimulates expression of the anti-apoptotic BCL-2 gene, resulting in the excess production of BCL-2 protein (McDonnell and Korsmeyer, 1991). The BCL-2 protein, located in the inner mitochondrial membrane, prolongs the survival of certain cells by preventing apoptosis (McDonnell and Korsmeyer, 1991). The overexpression of a structurally intact and functional BCL-2 anti-apoptotic protein leads to accumulation of inappropriately rescued long-lived B cells. This survival advantage likely predisposes cells with the BCL-2 mutation to further DNA damage (Biagi and Seymour, 2002). Thus, the t(14;18) translocation appears to be a necessary, but not sufficient, step for transformation of the cell to a malignant state (Limpens et al., 1991; Sehn, 2006).

The application of sensitive PCR techniques led to the discovery of detectable t(14;18) occurrences in the lymphocytes and lymphatic tissues of healthy (cancer-free) individuals (Limpens et al., 1991; Schuler et al., 2003). Though the clinical significance of the t(14;18) translocations found in healthy subjects remains unclear, those with a large number of circulating cells with the t(14;18) would be expected to have a higher risk of developing lymphoma compared to those with fewer such cells,

given that the t(14;18)-containing cells would be more likely to sustain the necessary additional genetic changes (Fuscoe et al, 1996). Several possible risk factors for t(14;18) prevalence and frequency in healthy individuals have been suggested. Variables found to influence t(14;18) prevalence include race (with Asians having a lower risk), age (with older individuals at increased risk), fair hair color, and light eye color (Yasukawa et al., 2001; Schüler et al., 2003; Baccarelli et al., 2006; Nambiar and Raghavan, 2010). Frequency of the chromosomal translocation has been found to be positively associated with age (Liu et al., 1994; Schüler et al., 2003), smoking (Schüler et al., 2003; Baccarelli et al., 2006), agricultural pesticide usage (Schroeder et al., 2001; Roulland et al., 2004; Agopian, 2009), Hepatitis-C virus infection (Zignego et al., 2002), exposure to ultraviolet B from sunlight (Bentham et al., 1999), and plasma TCDD levels (Baccarelli et al., 2006).

The major aim of the present study was to identify demographic, behavioral, clinical, and/or environmental factors that might contribute to the generation and/or expansion of t(14;18) translocations in the PBMCs of healthy individuals sampled from a residential city population located in Eastern Europe. Preliminary analysis of blood serum samples in this group revealed low TCDD levels with relatively high concentrations of several PCB congeners, both dioxin-like and non-dioxin-like. Thus, a secondary objective in this study was to assess the association between DL-PCB exposure and NHL risk in a population-based sample, presumably with a background exposure profile similar to that of the local populace. We hypothesized a dose-response relationship between serum levels of DL-PCBs and the chromosomal translocation. Relative expression of the BCL2 gene was obtained for a subsample of the blood specimens. We further posited a positive association between serum DL-PCB levels and BCL2 gene expression. A better understanding of the risk factors for t(14;18) prevalence and frequency would be useful in the characterization of those at risk of developing specific forms of NHL. Moreover, an association between DL-PCB exposure and the chromosomal translocation would represent a plausible

mechanistic step in NHL development, reinforcing previously observed associations between dioxin exposure and NHL.

B. Methods

1. Study participants

Dioxin exposure is not limited to those with occupational contact or to those living around point sources of dioxin pollution. The general population is exposed to dioxins as congener mixtures via widespread ambient pollution and the consumption of high-fat foods, including dairy goods, eggs, animal fat, and some types of fish (IARC, 1997). Our research team recruited 150 individuals with no known occupational or point source dioxin exposure from the general population of a city located in Eastern Europe. Eligible study subjects were selected from a local health insurance database. These subjects were originally assembled to act as a comparison group in studies of a cohort of chemical workers occupationally exposed to dioxins in a nearby city. They were frequency-matched to the occupationally exposed group on age, gender, ethnicity, and employment status. In a separate analysis, our research team is investigating the relationship between serum TCDD levels and t(14;18) prevalence and frequency in the chemical worker cohort. Only the population-based sample of 150 residents was considered in the present analysis.

Detailed medical histories were obtained for each subject, and qualified professionals performed physical examinations and collected blood samples for serum dioxin and biomarker studies. Survey information was entered into a database by the UIC Data Management Center in Kyiv, Ukraine and subsequently transferred to the UIC in Chicago, Illinois. Blood was drawn from individuals in the population-based study group during recruitment in 2011. These samples were immediately cryopreserved and stored at -80°C, then transferred in dry ice to partner laboratories at the CDC, Griefswald University in Germany, and the University of Milan in Italy. This study was approved by the CDC's NIOSH and the UIC Institutional Review Boards.

2. **Serum dioxin levels**

The CDC analyzed a 50 mL archived serum sample from each study participant for 7 PCDDs, 10 PCDFs, 4 coplanar PCBs (c-PCBs, including PCB 77, 81, 126, and 169), and 38 ortho-substituted PCBs by high-resolution gas chromatography/isotope-dilution high-resolution mass spectrometry (HRGC/ID-HRMS) Method 28 (Patterson et al., 1987). All dioxin analyses were conducted at the same testing facility with the same assay, equipment, and standardized procedures. Total lipid content of each specimen was estimated using a ‘‘summation’’ method (Akins et al., 1989), and analytical results were reported on a lipid-adjusted basis as pg/g lipid ppt for PCDDs, PCDFs, and c-PCBs, and as ng/g lipid ppb for ortho-substituted PCBs. Congener-specific detection limits were corrected for sample weight and analyte recovery. For individual analytes below the limit of detection (LOD), a value of zero was assigned. Total toxic equivalents were calculated according to the WHO 2005 relative toxic potency ranking scheme (Van den Berg et al., 2006). The geometric mean (and 95% confidence intervals[Cis]) serum levels for two dioxins (TCDD, PCB 126) and two representative NDL-PCBs (PCB 153, PCB 180) in this cohort were compared to serum levels in the general US population, as reported for 2003–2004 using data from the National Health and Nutrition Examination Survey (NHANES).

3. **Chromosomal translocations**

Standard operating procedure for the detection of t(14;18) translocations was carried out by Greifswald University in Greifswald, Germany. Whole blood was collected in two EDTA-vacutainer tubes, each 7 mL, and the blood was poured into leucosep tubes prefilled with Ficol solution; PBS was added to buffer up to a total volume (Ficoll + blood + PBS) of 45 mL per tube. Mononuclear cells were separated by Ficoll Hypaque density gradient centrifugation at 1,000 g for 10 minutes. The buffy coat containing the mononuclear cells was resuspended in 45 mL PBS and centrifuged at 300 g for 5 minutes; the pellet was resuspended again in 45 mL PBS. After centrifugation at 300 g for 5 minutes, the cell

pellet was resuspended in 1 mL PBS and the cell count was determined. After centrifugation, cell pellets (supernatant liquid removed) containing at least $1X10^7$ cells in 1.5 mL centrifuge tubes were stored in at least 2 aliquots at -80°C.

Quantitative real-time PCR was performed to detect the t(14;18) in PBMCs , as described previously by Dölken et al. (1998), using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). The DNA was obtained from roughly 140,000 cells, based on spectrophotometric measurements. As a reference, PCR of wild-type K-ras was performed. The reporter dye FAM (6-carboxy-fluorescein) and the quencher dye TAMRA (6-carboxy-tetramethylrhodamine) were used to label probes at the 50-end and 30-end, respectively. The PCR mix was made up of 400 nM of forward and reverse primers, 200 nM of probe, the TaqMan Universal PCR Master Mix (PE Applied Biosystems), and 0.1 to 2.0 µg of DNA. This mixture was incubated for 2 minutes to allow cleavage by Uracil-N-Glycosylase (UNG), followed by activation of AmpliTaq Gold by incubation at 95°C for 10 minutes. The denaturation step for each PCR cycle was set for fifteen seconds at 95°C, with the annealing/extension step of each cycle set for one minute at 61°C.

For detecting t(14;18), the standard operating procedure was carried out with five PCR reactions, using one µg of DNA, two positive controls, and two negative controls. Positive reference standards of t(14;18)-positive cell line Karpas 422 cloned fragments (200 or 700 copies per assay) were employed. Three PCR reactions using 0.1 µg of DNA were run to detect wild-type K-ras, with two positive and two negative controls. Aliquots of cloned wild-type K-ras (1,000 copies/assay) were used as positive reference standards for these PCRs. Additional DNA was examined when negative results were obtained for those groups of five aliquots that did not contain at least 350,000 cells.

The PCR technique described above has a sensitivity of one copy in 1–2 µg of DNA, or the ability to detect one t(14;18)-positive cell in 140,000–280,000 normal cells (Dölken et al. 1998).

4. **Gene expression analysis**

The PAXgene Blood RNA Kit was used to isolate total RNA (Qiagen-PreAnalytix, Hombrechtikon, Switzerland) in a subset of the cohort (n=30). A sample from the occupational cohort discussed previously was selected for gene expression analysis for other studies. This sample was obtained by randomly selecting 20 subjects from each tertile of serum TCDD levels. These 60 subjects were subsequently stratified by age (<67.5, ≥67.5), gender, ethnicity (Caucasian versus other), and prevalence of t(14;18) (0 translocations versus >0 translocations). The 30 subjects from the population-based sample were randomly selected and matched to the occupationally exposed sample on these stratification variables. Qiagen prepared a customized array of 83 genes selected by our team for this study and all future studies of gene expression in the cohort. These candidate genes were chosen based on several criteria: (1) they were known to be key components of the AhR, human lymphoma, and apoptosis pathways, (2) they were shown to be upregulated greater than 1.30-fold or down-regulated at least 0.65-fold in previous studies of dioxin-exposed humans, or (3) their expression was shown to be changed 5-fold in previous studies of dioxin-exposed human cell lines. Among the genes selected was the BCL2. Complementary DNA was synthesized with a RT^2 First Strand kit (SABiosciences, Frederick, Maryland, USA) and analyzed with specific RT^2 Profiler PCR arrays by RT-PCR using Syber Green chemistry. The PCR array is a complete system for pathway-focused gene expression analysis with some components that guarantee high-quality, reproducible, and reliable gene expression data. The Profiler PCR array includes 89 RT^2 qPCR primer assays (including five housekeeping genes) and a proprietary control panel to investigate contamination with genomic DNA (gDNA), quality of the reverse transcription reaction, and real-time efficiency (Qiagen Inc., Mississauga, Canada). All RT-PCR runs were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, California, USA).

5. **Statistical analysis**

Outcome variables of interest were t(14;18) prevalence (proportion of subjects with the translocation), and t(14;18) frequency for subjects who had at least one detectable translocation per million cells. Risk factors for prevalence (those that cause chromosomal breaks and translocations) are likely to be different than those that increase the frequency of translocations (e.g., by preventing apoptosis). As such, the analysis of t(14;18) prevalence (which includes those with and without detectable translocations) was performed to determine whether a risk factor might cause the initiation of a translocation, whereas the analysis of t(14;18) frequency addressed whether a risk factor might lead to an increase in the number of circulating translocations (i.e., translocation expansion). Since the purpose of the t(14;18) frequency analysis was to assess whether the mechanism of t(14;18) expansion could be distinct from the t(14;18)-initiation mechanism tested by the t(14;18) prevalence analysis, only subjects with at least one detectable translocation per million cells tested were included in the t(14;18) frequency analysis.

Several potential risk factors for increased t(14;18) prevalence and frequency were considered. Demographic variables assessed included age, gender, BMI, height, eye color, hair color, skin color (including proneness to sunburn), ethnicity (Caucasian, Asian, or other), and family history of hematopoietic cancer. Behavioral factors related to smoking habits included smoking status (current-, former-, or never-smoker); smoking duration (years); pack-years; and age at which the subject began smoking. Factors related to occupational and/or environmental exposures included blood levels of dioxins and NDL-PCBs, reported history of pesticide usage, and reported ultraviolet (UV) light exposure.

Contingency tables and χ^2 tests of association were used to test the bivariate relationship between various potential risk factors and t(14;18) prevalence. For the t(14;18) frequency variable, bivariate relationships were assessed by comparing measures of central tendency for the non-normal

outcome at different levels of the exposure variable. Wilcoxon Rank Sum tests were used to compare two groups, while Kruskall-Wallis tests were utilized for multiple-group comparisons.

Statistical models were fit to the data to obtain a least-biased estimate of the associations between each of the potential risk factors and t(14;18) prevalence and frequency. For models with t(14;18) prevalence (presence of at least one translocation versus no detected translocations) as the outcome of interest, unconditional logistic regression models (Breslow and Day, 1980)were applied to estimate exposure-specific odds ratios and 95% CI for each independent risk factor. Risk factors for t(14;18) frequency outcome variables were assessed using Poisson regression techniques to model t(14;18) rates (number of translocations per million cells).

A backward elimination strategy was considered for inclusion in the final risk model, and product terms were included to test for statistical interactions between covariates (Kleinbaum et al., 2007). Likelihood ratio tests were employed to compare nested models (Rothman et al., 2008) and identify significant risk factors. A priori knowledge was also considered for inclusion in the final model, and all models were adjusted for age, gender, and ethnicity.

As discussed previously, the t(14;18) would be expected to produce an increase in BCL2 gene expression. Thus, high-risk groups, as defined by the risk factor regression analysis, would be expected to have higher levels of BCL2 expression when compared with low-risk groups. To test this, subjects were split into high- and low-risk groups based on model prediction of prevalence (present versus not present) and high versus low frequency for those with greater than 0 translocations. To test for a dose-response relationship with BCL2 expression, subjects were grouped by risk tertile for the t(14;18) frequency outcome variables. Relative BCL2 expression levels were determined using the ΔΔCt method (Livak and Schmittgen, 2001). Statistical comparisons by risk group were performed using t-tests, accounting for the false discovery rate (FDR) using QVALUE software written by Alan Dabney and John

Storey (Storey, 2002). All statistical analyses were performed using SAS 9.3 (Cary, North Carolina, USA) or QVALUE.

C. Results

1. Translocation prevalence and frequency

Compared to the general US population, as reported using the NHANES 2003–2004 sample cycle, the study cohort had comparable TCDD serum levels (Table III). However, levels of DL-PCB 126 (ppt, lipid-adjusted) were almost four times as high in the study group. Serum levels of NDL-PCB 153 and PCB 180 in the cohort members were also found to be moderately elevated when compared to the NHANES population. All measurements for these specific congeners in the study sample were above the limit of detection.

TABLE III

COMPARISON OF BLOOD SERUM DIOXIN AND POLYCHLORINATED BIPHENYL CONCENTRATIONS IN A POPULATION-SAMPLED COHORT AND THE NHANES POPULATION SAMPLE

	Geometric mean[a]	LCL	UCL
2,3,7,8-TCDD[b]			
NHANES	< 3.8	N/A[d]	N/A[d]
Cohort	2.5	2.3	2.7
PCB 126[b]			
NHANES	16.3	14.9	17.9
Cohort	60.7	53.2	69.3
PCB 153[c]			
NHANES	19.8	18.8	20.9
Cohort	51.7	47.6	56.1
PCB 180[c]			
NHANES	15.1	14.5	15.7
Cohort	20.9	19.2	22.6

[a] Study subjects sampled in 2009;
NHANES population sampled 2003–2004.

[b] Measured in ppt, lipid-adjusted.

[c] Measured in ppb, lipid-adjusted.

[d] Not calculated due to proportion of results below the LOD: LOD = 3.8 ppt.

The t(14;18) translocation was detected in 75 (50%) of the 150 subjects included in this study. None of the potential risk factors considered were found to be significantly associated with t(14;18) prevalence (presence versus absence of a detected translocation). Table III shows the prevalence of the chromosomal aberration by level of selected variables of interest. A higher proportion of subjects with a detectable t(14;18) translocation had light eyes compared to dark eyes, though this difference was not statistically significant (p=.18). Prevalence was also higher in subjects who reported that their skin burns after one hour in the sunlight, compared with those who reported that their skin never burns, though this result was not statistically significant (p=.086). A larger proportion of light-skinned (51%) versus

dark/olive-skinned (38%) subjects had detectable t(14;18) translocations, though there were relatively few dark-skinned individuals in the study sample (n=8). No statistically significant differences in t(14;18) prevalence were seen in subjects with light hair (44%) versus dark hair (53%). Associations with eye, hair, and skin color, as well as with proneness to sunburn, remained nonsignificant after adjustment for age, gender, and ethnicity in logistic regression models.

TABLE IV

T(14;18) PREVALENCE AND FREQUENCY IN A POPULATION-SAMPLED COHORT

		t(14;18)-positive subjects	t(14;18) frequency	
	N (%)	%	Mean[a]	(95% CI)
Gender				
Male	76 (50.7)	48.7	3.58	(2.42–5.30)
Female	74 (49.3	51.4	3.99	(2.96–5.38)
Ethnicity				
Caucasian	75 (50.0)	48.0	3.96	(2.84–5.51)
Asian	59 (39.3)	52.1	3.70	(2.55–5.35)
Other	2 (1.3)	50.0	1.80	–
Age				
≤ 67.5	75 (50.0)	52.0	3.57	(2.67–4.77)
> 67.5	75 (50.0)	48.0	4.03	(2.69–6.04)
Body mass index				
< 25	29 (19.3)	41.4	3.73	(2.20–6.34)
25-29	59 (39.3)	52.5	3.34	(2.25–4.96)
≥ 30	48 (32.0)	50.0	5.07	(3.11–8.25)
Missing	14 (9.3)	57.1	2.61	(1.40–4.87)
Smoking Status				
Current	31 (20.7)	54.9	2.88	(2.73–4.76)
Former	29 (19.3)	41.4	6.70[c]	(2.78–16.14)
Never	90 (60.0)	51.1	3.60	(1.75–4.75)
Pack years				
≤ 20 years	17 (29.3)	47.1	5.40	(2.19–13.3)
> 20 years	41 (70.7)	48.8	3.08	(1.92–4.91)
Eye color				
Light	88 (58.7)	54.6	3.13	(2.38–4.13)
Dark	62 (41.3)	43.6	5.28	(3.36–8.31)
Hair color				
Light	18 (12.0)	44.4	2.88	(1.22–6.79)
Dark	98 (65.3)	53.1	3.40	(2.61–4.44)
Unspecified	34 (22.7)	44.1	6.32	(3.16–12.63)
PCB 126 blood level[b]				
≤ 40.2	50 (33.3)	48.0	3.09	(2.16–4.40)
40.3-81.5	49 (32.7)	46.9	3.17	(2.25–4.46)
> 81.5	51 (34.0)	54.9	5.22	(3.14–8.66)

[a] Geometric mean and 95% CI for t(14;18)-positive subjects.

[b] Measured in parts per trillion (ppt), lipid adjusted.

[c] $p<.05$.

The only statistically significant crude increase in t(14;18) frequency was observed in former smokers (p=.02), as shown in Table IV. Mean t(14;18) frequency was nonsignificantly elevated in obese subjects (p=.38) and in subjects with relatively higher PCB 126 blood serum levels (p=.06). Among smokers, there were no significant associations between t(14;18) frequency and smoking duration or pack years. Only two subjects reported having a family history of leukemia, whereas no subjects reported a family history of lymphoma.

Poisson regression models were fit to the data for cohort subjects who had at least one translocation detected (n=75). A main effects model (without interaction terms) produced relative risk (RR) estimates of 1.01 (95% CI: 0.98–1.04) for each 10 ppt increase in PCB 126 serum levels, 4.29 (95% CI: 1.98–9.28) for former smokers compared with never-smokers, and 1.07 (95% CI: 0.42–2.74) for current smokers compared with never-smokers, after adjustment for age, gender, and ethnicity. The BMI could not be calculated for eight subjects with detectable t(14;18) translocations, and inclusion of BMI did not improve model fit. Thus, to maximize sample size for effect estimation, BMI was excluded from the final model. Cigarette pack years did not correlate with t(14;18) frequency in the study group as a whole. When joint effects were considered (using product terms in the model), a significant interaction between smoking status and t(14;18) frequency became apparent. Figures 5 and 6 show scatterplots of PCB 126 blood serum levels versus t(14;18) frequency (on a log scale) for smokers (current and former) and never-smokers, respectively. The geometric mean serum PCB 126 concentration was 87.1 ppt (range: 24.0–678.0 ppt) in nonsmokers and 60.0ppt (range: 22.3–165.0 ppt) in former smokers. The geometric mean serum PCB 126 level in current smokers was lower at 27.0 ppt (range: 11.0–87.8 ppt). According to the regression model, each 10-ppt increase in PCB 126 blood serum levels (ppt) changed the rate of t(14;18) translocations per million cells by a factor of 0.99 (95% CI: 0.95–1.03) in never-smokers, 1.13 (95% CI: 1.03–1.24) in former smokers, and 1.51 (95% CI: 1.17–1.94) for current smokers (Table V). When the relationship between PCB 126 blood serum levels and t(14;18)

frequency was modeled for current and former smokers alone, pack years became a statistically significant predictor having a positive correlation with translocation frequency (data not shown).

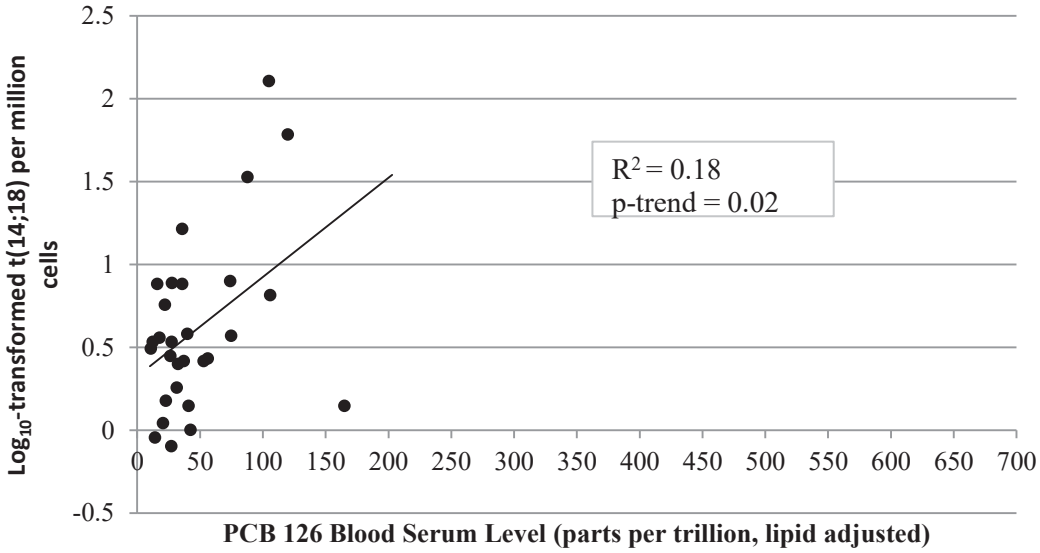

Figure 5. Scatterplot of polychlorinated biphenyl 126 (PCB 126) blood serum levels and t(14;18) translocations among current and former smokers.

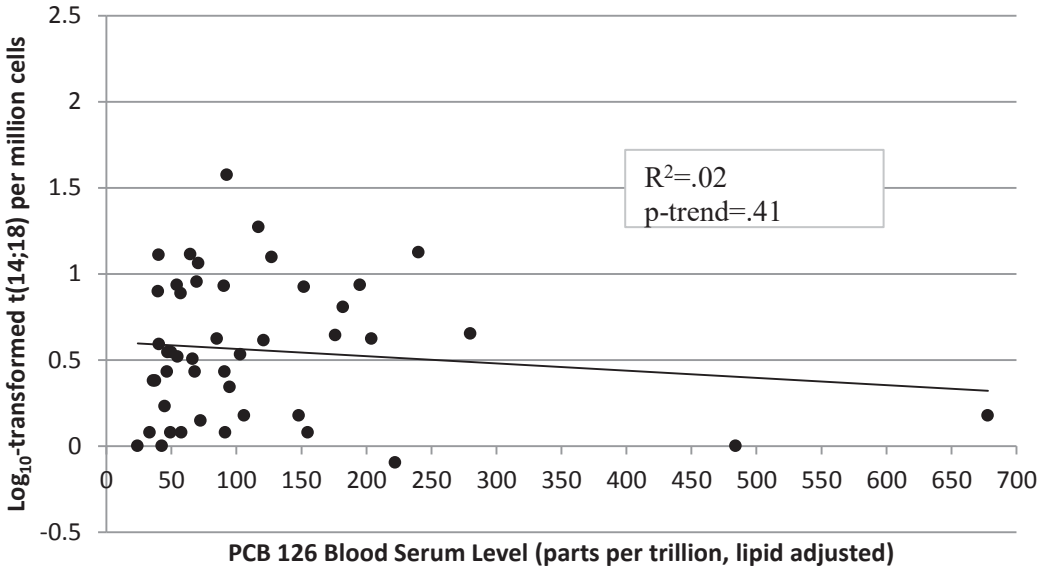

Figure 6. Scatterplot of polychlorinated biphenyl 126 blood serum levels and t(14;18) translocations among never-smokers.

TABLE V

MODELS OF THE EFFECTS OF PCB126 BLOOD SERUM LEVELS ON T(14;18) FREQUENCY AND RELATIVE BCL2 EXPRESSION

	Relative Risk[a,b]	95% CI
Cohort[d]		
Current Smoker	1.51	1.17–1.94
Former Smoker	1.13	1.03–1.24
Never Smoker	0.99	0.95–1.03
	Fold Change[a,c]	**95% CI**
Subsample[e]		
Smoker (Former/Current)	1.09	1.04–1.14
Nonsmoker	1.02	1.01–1.03

[a] Models adjusted for age, gender, and ethnicity.

[b] Poisson joint-effects model; Relative risk [t(14;18) translocations per 1 million cells] associated with a 10-unit increase in serum PCB 126 level (ppt, lipid-adjusted).

[c] Linear regression joint-effects model; Fold change (relative BCL2 expression) associated with a 10-unit increase in serum PCB 126 level (ppt, lipid-adjusted); Fold change calculated as 2(-ΔΔCt).

[d] Includes cohort subjects with >0 translocations (n=75).

[e] Includes subsample with >0 translocations (n=16).

2. **Gene expression**

Relative expression of the BCL2 gene was quantified in a subsample of 30 subjects from the study group. As shown in Table VI, BCL2 expression was 1.6-fold higher in subjects with PCB 126 blood serum levels between 38.7 ppt and 73.3 ppt (second tertile) compared to those with less than 38.7 ppt (reference group). Expression was 1.8-fold higher in subjects with PCB 126 blood serum levels greater than 73.3 ppt (highest tertile) compared to the reference group.

Risk differences estimated from multivariable linear regression models for subjects in the subsample who had at least one detectable t(14;18) translocation (n=16) are presented as fold change differences in BCL2 expression in Table V. After adjustment for age, gender, and ethnicity, blood serum levels of PCB 126 were found to have a statistically significant interactive effect with smoking status (current/ever versus never). Specifically, the model suggests that the normalized cycles to threshold (ΔCt) decreased by 0.02 cycles with each 10-ppt increase in serum PCB 126 levels in never-smokers. This corresponds to a fold change of 1.02 (95%CI: 1.01–1.03). In smokers, each 10-ppt increase in serum PCB 126 levels decreased the ΔCt by 0.12 cycles, which corresponds to a fold change of 1.09 (95%CI: 1.04–1.14).

TABLE VI

RELATIVE EXPRESSION OF THE BCL2 GENE BY TERTILES OF BLOOD SERUM PCB 126 CONCENTRATION IN A POPULATION-SAMPLED COHORT

Gene	PCB 126[a]	n	mean ΔCt[b]	std. dev.	median ΔCt	min	max	Fold change[c]	p-value[d]
BCL2	≤ 38.6	10	4.5	0.3	4.5	4.0	4.9	-	
	38.7–73.3	11	3.9	0.7	3.8	2.5	5.0	1.6	0.007
	> 73.3	9	3.6	0.7	3.8	2.4	4.5	1.8	

[a] PCB 126 blood serum levels measured in ppt; cutoffs chosen based on tertile.

[b] ΔCt = difference in cycles to threshold (number of PCR cycles necessary to reach threshold expression level).

[c] Fold change calculated as $2^{(-\Delta\Delta Ct)}$.

[d] Linear regression of BCL2 relative expression on PCB 126 serum level tertiles used to obtain p-value.

D. **Discussion**

Blood serum levels of both DL- and NDL-PCBs in this study cohort of 150 population-selected subjects were found to be elevated relative to those of individuals in the general US population (as estimated from the 2003–2004 NHANES cycle). Of note, the geometric mean of PCB 126 concentrations in the study sample was measured at 60.7 ppt (95% CI: 53.2–69.3), compared to 16.3 ppt (95% CI: 14.9–17.9) in those aged 20 and older in the general US population (CDC, 2009). Blood levels of TCDD in the study population sample were comparable to those of the general US population. Though all dioxins share the same mechanism of action, the toxic potency of each congener is unique. As a result, the WHO developed a relative toxic potency ranking scheme, assigning a TEF to each compound. The TEF represents the order of magnitude by which the compound's toxic potency differs from that of the most toxic compound, TCDD. It typically represents the ratio of TCDD's ED_{50} to the ED_{50} of the dioxin of interest. As such, the TEF is equal to 1.0 for TCDD and less than 1.0 for all other dioxins (with the exception of 1,2,3,7,8-pentachlorodibenzo-p¬-dioxin, which also has a TEF of 1.0) (Van den Berg et al., 2006; White and Birnbaum, 2009). Experimental evidence classifies PCB 126 as the most toxic DL-PCB—this congener was assigned a TEF of 0.1 (Van den Berg et al., 2006). The elevated average levels of PCB 126 in our study cohort provided a unique opportunity for an assessment of the role that DL-PCB exposure might play in t(14;18) production and expansion.

We did not identify any factors that were statistically associated with t(14;18) prevalence, though a greater proportion of individuals with light eyes (but not light hair) and those with a self-reported tendency to sunburn had a detectable number of these chromosomal abnormalities in their lymphocytes. An assessment of t(14;18) prevalence and frequency in a sample of Seveso, Italy, residents environmentally exposed to TCDD noted an increase in t(14;18) prevalence among those with light eyes and light hair (Baccarelli et al., 2006). Our observed lack of association may have resulted from lack of appropriate sample size or differences between our Eastern European cohort and other studied cohorts

in UV exposure intensity. However, it has also been suggested that the t(14;18) translocation might be detected in all healthy individuals, given a high enough test sensitivity (Hirt et al., 2007; Schuler et al., 2009). Thus, differences in testing procedures might also have impacted our estimates of prevalence. Baccarelli et al. (2006) detected a t(14;18) translocation in the peripheral blood lymphocytes of 50 out of 144 (34.7%) subjects evaluated, whereas we discovered this chromosomal aberration in 75 out of 150 (50%) subjects. For further comparison, in their assessment of a group of 715 healthy individuals aged 0 to 91, Schüler et al. (2009) detected the t(14;18) in 46% of study subjects using a similar standardized quantitative real-time PCR assay. However, for those aged 50 and older (similar to the age distribution in our study), the t(14;18) was identified in 58%.

Among the subjects in our study in whom chromosomal translocations were detected, the frequency ranged from 0.8 to 127.2 t(14;18)-positive cells per million cells tested. Based on their review of studies that analyzed the peripheral blood of healthy individuals, Schüler et al. (2003) estimated an average range of 0.1–100 t(14;18)-positive cells per million cells. Thus, the frequency range of chromosomal translocations identified in our study was comparable to that of others.

In the crude analysis of t(14;18) frequency, former smokers had a statistically higher number of translocations per million cells than nonsmokers, though a similar association was not observed in current smokers. An association between smoking behavior and t(14;18) frequency has not been definitively established in the literature. Two studies reported increased translocation frequency in healthy individuals (Bell et al., 1995; Baccarelli et al., 2006), whereas a large population-based study carried out in Germany failed to identify any significant relationships between t(14;18) frequency and several measures of smoking behavior (Hirt et al., 2013). In our study, we observed different mean concentrations of serum PCB 126 in never-, former-, and current-smokers. Specifically, current smokers were found to have the lowest serum PCB 126 levels.

Our results suggest that PCB 126 exposure may contribute to the expansion of t(14;18) frequency in human lymphocytes. Further, this relationship was only observed in those with past and/or present exposure to constituents in cigarette smoke. This association was supported by the observed interaction between serum PCB 126 levels and smoking on the relative gene expression of BCL2, which would be expected to be upregulated in those with a larger number of t(14;18) translocations. In a study of Seveso residents, who experienced relatively large dioxin exposures, the frequency of t(14;18) translocations was found to increase in a dose-response fashion with increasing serum dioxin levels. These researchers investigated the relationship between translocation frequency and serum levels of the most potent dioxin, TCDD. Environmental exposures to TCDD in Seveso were estimated to be very high. In their study, smoking duration was also associated with expansion of t(14;18) clones (Baccarelli et al., 2006). Though smoking has not been established as a strong risk factor for NHL in general, evidence suggests a significant association between smoking and follicular lymphoma in particular (Morton et al., 2005). The significant interaction between serum PCB 126 levels and smoking status in our study may shed light on some of the more subtle actions of mixed exposures at closer-to-ambient levels.

Dioxins exert their toxic effects primarily through persistent activation of the AhR pathway. More toxic dioxins, such as TCDD, bind to the AhR with higher affinity (White and Birnbaum, 2010). Similarly, cigarette smoke exposure has been shown to activate the AhR. Several constituents of cigarette smoke, which include dioxins and PAHs, were found to act cooperatively in the stimulation of this pathway (Kitamura and Kasai, 2007).

The t(14;18) translocation is believed to occur as a result of erroneous recombination of variable, diversity, and joining genes of the immunoglobulin heavy chain (IgH) locus during the early phases of B-cell maturation (Tsujimoto et al., 1985). Though the mechanisms involved presently remain uncharacterized, B-cells appear to be sensitive to dioxin-mediated activation of the AhR pathway.

Several murine models have revealed the impairment of B-cell to plasma cell differentiation with prolonged AhR activation (Sulentic and Kaminski, 2011). This process involves several transcription factors (Nguyen et al., 2013). Further investigation into these pathways may provide useful insight into the mechanisms through which dioxin-like compounds and cigarette smoke exposures might trigger chromosomal translocations.

Furthermore, a group of researchers recently investigated the interaction between smoking behavior and exposures to dioxins. In one study, they found an interactive effect of smoking status and serum levels of various persistent organic pollutants (POPs) on all-cause mortality in a group of elderly (≥ 70 years old) NHANES participants. Specifically, they estimated a significant interaction between smoking status and PCB 126 serum levels (Lee et al., 2013). Using a different dataset (the Prospective Investigation of the Vasculature in Uppsala Seniors), these researchers again demonstrated a lower risk of mortality in elders with high serum levels of persistent organic pollutants who were never smokers as compared to smokers (Lee et al., 2014).

1. **Limitations**

Though we believe that this study provides important insight into the interaction of exposure to dioxin-like compounds and smoking habits, there were limitations that must be acknowledged. First, given their lipophilic structure, many of the dioxins are known to be highly biopersistent (ATSDR, 1998). As a result, we were unable to draw any conclusions regarding the impact of the timing of PCB 126 exposure. However, our results do indicate that past smoking habits can still have an impact on the relationship between serum PCB 126 levels and t(14;18) frequency.

Second, as discussed previously, the study sample used in this analysis was frequency-matched on age, among other factors, to an occupational cohort investigated in other studies. The median age in our population-based group was 67.5, with no adults younger than 50 years of age. Thus, we were

unable to fully account for the expansion of t(14;18) that has been found to occur as a result of aging (Schüler et al., 2009).

Third, our results should be interpreted with caution, given the relatively low n-size of the study. Analyses of t(14;18) frequency differences were based only on those in whom at least one t(14;18) was detected (n=75). Our gene expression analysis, which was based on a subsample of cohort members, was similarly limited (n=16). However, our study seemed to have the power to detect significant differences in t(14;18) frequency between comparison groups, and these differences were striking.

2. Conclusions

Our results suggest that smoking habits may moderate the effects of PCB 126 exposure on chromosomal translocation frequency. Specifically, increasing serum PCB 126 levels were associated with expansion of t(14;18) translocations, which represent an early step in the progression toward the development of follicular lymphoma. Thus, we have demonstrated further support for the biological plausibility of previously observed links between exposure to dioxin-like compounds and NHL. Further, these findings have important implications for risk assessment. Though smoking is a well-known risk factor for a myriad of adverse health conditions, public health professionals and clinicians should continue to document and understand its ability to potentiate the adverse effects of other environmental and occupational exposures, with the goal of designing the most effective cessation interventions.

IV. EFFECT OF NON-DIOXIN-LIKE POLYCHLORINATED BIPHENYL EXPOSURE ON DIOXIN-INDUCED EXPRESSION OF THE ARYL HYDROCARBON RECEPTOR GENE IN HUMANS

A. Background

Dioxins, a family of toxic chemical contaminants, are now widespread in the environment. The dioxins are similar in chemical structure and share a common mode of action. Included in this group of substances are the PCDDs, the PCDFs, and a subcategory of DL-PCBs that share the same mode of action (IARC, 1997). Many of the effects of dioxins are moderated by the AhR. Several PCDDs, PCDFs, and PCBs share an affinity for binding with the AhR, and so are aptly referred to as dioxin-like. Currently, these include 7 PCDDs, 10 PCDFs, 4 non-ortho-PCBs, and 8 mono-ortho-PCBs. Though the mechanism of action is the same, the toxic potency of each substance is unique (ATSDR, 1998).

When not bound to a ligand, the AhR resides in the cell cytoplasm, where it interacts with several chaperone proteins. These include two Hsp 90s, one prostaglandin E synthase 3 (p23) protein, and one immunopilin-like protein hepatitis B virus XAP2. After diffusion into the cell, dioxins bind the AhR, which then releases one molecule of Hsp90 as well as both p23 and XAP2. The AhR-dioxin complex next translocates into the nucleus, where it heterodimerizes with the ARNT at their respective PAS domains. After the release of the remaining molecule of Hsp90, the AhR-dioxin-ARNT complex binds to DNA at dioxin response elements in the promoter region of target genes to initiate transcription. The ability of the complex to initiative transcription may be altered by coactivators (e.g., thyroid hormone receptor/retinoblastoma protein-interacting protein 230) or corepressors (e.g., estrogen receptor alpha) (White and Birnbaum, 2010).

Dioxins exert their toxicity by persistently activating the AhR and thus preventing it from performing its normal homeostatic functions. The AhR appears to be necessary but not sufficient for most dioxin-associated toxicity (White and Birnbaum, 2010). It may play a role in cell-cycle regulation and the suppression of tumors in various organs and tissues (Marlowe and Puga, 2005; Singh et al., 2008). Thus, disruption of normal functioning could potentially lead to adverse outcomes. Persistent activation of the AhR has been shown to affect processes related to cell proliferation, adipose differentiation, apoptosis, tumor promotion, and immune and endocrine function (Abel and Haarmann-Stemmann, 2010). It may also lead to increased competition for heterodimerization with the ARNT, displacing other potentially important signaling partners (White and Birnbaum, 2010).

The AhR-dioxin-ARNT complex stimulates the transcription of several genes, especially those coding for xenobiotic metabolizing enzymes. These include cytochrome P450 enzymes (CYPs), aryl hydrocarbon hydroxylase, and ethoxyresorufin o-deethylase (Safe, 1989; Nagao, 1990). Activation of the AhR also stimulates the AhRR, a negative regulator of AhR activity. The AhR action induces gene and protein expression of the AhRR, indicating that AhR and AhRR form a regulatory feedback loop (Hahn et al., 2009; Kawajiri and Fukii-Kuriyama, 2007).

Humans typically come into contact with a diverse mixture of chemicals through environmental and/or occupational exposure. Because of the complex nature of these exposures, the study of the interaction between different toxic agents in the human body is crucial. Some data suggest that combined exposures to some dioxin-like congeners may have antagonistic (rather than additive) effects, and that these effects may be species-specific (EPA 2010). There is also evidence that NDL-PCBs may exhibit antagonistic activity for some responses (Safe, 1998). Several NDL-PCBs have been identified in experimental studies to have antagonistic effects on dioxin-mediated response (Bannister et al., 1987; Haake et al., 1987; Biegel et al., 1989; Davis and Safe, 1989). It has been suggested that under conditions where TCDD elicits a submaximal AhR-mediated response, the presence of a second full agonist will

enhance this response. On the other hand, under conditions where TCDD elicits a maximal response, a partial agonist or antagonist compound will inhibit the response through unproductive AhR binding. It would seem that various planar, nonpolar molecules can bind the AhR but differ in their ability to trigger conversion to the DNA-binding form. While coplanar PCBs (e.g., PCB 77, PCB 126) and mono-ortho PCBs (e.g., PCB 156) have been shown to act as agonists of the TCDD response, as measured by increased CYP1A1 gene transcription and protein levels, non-planar PCBs (e.g., PCB 153) have been observed to act antagonistically (Chen and Bunce, 2004).

The purpose of this study was to assess whether the dioxin-induced expression of the AhR pathway genes might be attenuated by concurrent NDL-PCB exposure in humans. Expression of mRNA was examined in a cohort of chemical factory workers with past occupational exposure to dioxins and in a comparison group from a nearby city presumably without sizable environmental dioxin contamination. Both study groups exhibited a range of serum NDL-PCB levels. We posited that antagonistic effects on AhR and AhR-mediated gene expression would be detectable in a human study population. Our results may have important implications for the risk assessment of populations potentially exposed to dioxins and PCBs.

B. **Methods**

1. **Study participants**

In 2009, 218 former workers with historical occupational exposure to dioxins while engaged in the manufacture of chlorinated phenols and their phenoxy-acid esters were recruited. The chemical plant where these subjects worked, located in Eastern Europe, was active from the early 1940s until 2004 and produced various chlorinated chemicals, from household bleach to pesticides. Emissions discharged by toxic waste incinerators at the plant contaminated the surrounding city with dioxins. For comparison, 150 additional individuals with comparable demographics from the general population of a

city located approximately 250 kilometers from the chemical plant were enrolled. Factory workers and subjects in the population-based sample were matched on age, gender, ethnicity, and employment status.

The factory workers included in this study were involved in the production of TCP and TCP-Cu, 2,4,5-T, and 2,4-D. Detailed information regarding each worker's occupation at the plant was obtained from plant personnel records and a survey completed by each study subject. Surveys were administered to collect demographic (e.g., age, ethnicity), behavioral (e.g., smoking status), and medical (e.g., previous diagnoses, family history of cancer) information from each worker and control subject. Qualified professionals performed physical examinations and collected blood samples for serum dioxin and biomarker studies. The subset of medical and occupational history data that were related to study hypotheses were entered into a database by the UIC Data Management Center in Kyiv, Ukraine, and subsequently transferred to the UIC. After they were drawn, blood samples from the 218 exposed and 150 unexposed participants were immediately cryopreserved and stored at -80°C, then transferred in dry ice to partner laboratories at the CDC, Griefswald University in Germany, and the University of Milan in Italy.

A subset of these study participants was selected for gene expression analysis. Specifically, a stratified random sample of 60 exposed subjects, based on tertiles of serum TCDD level, was selected from the population of 218 exposed subjects for whom RNA samples were archived. Also selected was a random sample of 30 unexposed subjects from the 150 population-selected participants, frequency-matched to the 60 exposed subjects on age, gender, ethnicity, and presence/absence of detectable t(14;18) translocations (these chromosomal aberrations were of interest in our other studies of these cohorts). Qiagen prepared a customized array for 84 genes selected by our team. The criteria for selecting candidate genes were: (1) that they were key components of the AhR, human lymphoma, or apoptosis pathways; (2) that they showed >1.30-fold upregulation or 0.65-fold down-regulation in

previous studies of dioxin-exposed humans; or (3) that they showed a >5-fold change in expression in previous studies of dioxin-exposed human cell lines. This study was approved by the CDC NIOSH and the UIC Institutional Review Boards.

2. **Serum dioxin levels**

The CDC analyzed a 50 mL archived serum sample from each study participant for 7 PCDDs, 10 PCDFs, 4 coplanar PCBs (c-PCBs, including PCB 77, 81, 126, and 169), and 38 ortho-substituted PCBs by high-resolution gas chromatography/isotope-dilution high-resolution mass spectrometry (HRGC/ID-HRMS) Method 28 (Patterson et al., 1987). All dioxin analyses were conducted at the same testing facility with the same assay, equipment, and standardized procedures. Total lipid content of each specimen was estimated using a summation method (Akins et al., 1989), and analytical results were reported on a lipid-adjusted basis as pg/g lipid ppt for PCDDs, PCDFs, and c-PCBs, and as ng/g lipid ppb for ortho-substituted PCBs. Congener-specific detection limits were corrected for sample weight and analyte recovery. For individual analytes below the LOD, a value of zero was assigned. Total toxic equivalents were calculated according to the WHO 2005 relative toxic potency ranking scheme (Van den Berg et al., 2006). The geometric mean (and 95% CIs) serum levels for two dioxins (TCDD, PCB 126) and two representative NDL-PCBs (PCB 153, PCB 180) in this cohort were compared to serum levels reported for 2003–2004 in the general US population (NHANES).

3. **Gene expression analysis**

The PAXgene Blood RNA Kit was used to isolate total RNA (Qiagen-PreAnalytix, Hombrechtikon, Switzerland). Complementary DNA was synthesized with a RT2 First Strand kit (SABiosciences, Frederick, Maryland, USA) and analyzed with specific RT2 Profiler PCR arrays by RT-PCR using Syber Green chemistry. The PCR array is a complete system for pathway-focused gene expression

analysis with some components that guarantee high-quality, reproducible, and reliable gene expression data. The Profiler PCR array includes 89 RT2 qPCR primer assays (including 5 housekeeping genes) and a proprietary control panel to investigate contamination with genomic DNA (gDNA), quality of the reverse transcription reaction, and real-time efficiency (Qiagen Inc., Mississauga, Canada). All RT-PCR runs were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, California, USA), which generated the Ct for each gene of interest. The Ct represents the PCR cycle number at which the reaction fluorescence crosses a predetermined fluorescence threshold that is significantly above the background fluorescence level. The Ct represents a point at which the amplicon product becomes detectable during the early exponential phase of the reaction, and it is inversely proportional to the gene of interest's initial expression level (Qiagen Inc., Mississauga, Canada).

4. **Statistical analysis**

Relative expression levels of genes in the AhR pathway were determined using the ΔΔCt method. This method assumes 100% efficiency of the PCR process at threshold (i.e., amount of PCR product amplified is fully doubled with each cycle) (Livak and Schmittgen, 2001). The protocol for calculating relative expression levels for each gene of interest was standard. First, raw Ct values for the 5 housekeeping genes were averaged for each sample. Next, ΔCt values were calculated for each sample by subtracting the Ct values for the housekeeping genes from the Ct values for the genes of interest. Then, ΔCt values for biological replicates were averaged, and ΔΔCt values were calculated by subtracting the ΔCt for the low-risk group (i.e., unexposed references) from the ΔCt for the high-risk group (i.e., exposed workers). Finally, fold change (a measure of how many times more/less expressed a gene is in the high-risk group compared with the low-risk group) was calculated according to the formula: fold change = $2^{(-(\Delta\Delta Ct))}$.

Genes associated with the AhR pathway, including those regulated by the AhR (CYP1A1, CYP1A2, AhRR), were assessed for differential expression in the comparison groups. For comparison of the occupational cohort sample with the frequency-matched population control sample, raw ΔCt values were evaluated and statistical differences in expression were evaluated using the Welch-Satterthwaite t-test for comparisons of groups with unequal variances, accounting for the FDR using QVALUE software written by Alan Dabney and John Storey (Storey, 2002). For the comparison of subjects categorized within tertiles of total TEQ blood serum levels (ppt), multivariable linear regression models were employed to adjust for age, gender, and ethnicity.

Once genes in the AhR pathway that were significantly up- or down-regulated were identified, stratified analyses were carried out to determine whether concurrent blood levels of NDL-PCBs modified the association between dioxin exposure and AhR gene expression. The NDL-PCB congeners assessed included seven congeners (PCBs 28, 52, 101, 118, 138, and 180) that represent a group of indicator PCBs that the European Union Community Bureau of Reference recommended be monitored. These PCBs were chosen as indicators of NDL-PCB exposure based on their diversity (wide range of chlorination) and their relatively high concentrations in technical mixtures (Webster et al., 2013). All statistical analyses were performed using either SAS 9.3 (Cary, North Carolina, USA) or QVALUE.

C. Results

1. Expression of aryl hydrocarbon receptor pathway genes

Expression of selected genes related to the AhR pathway in the occupational cohort (exposed) and the population control group (unexposed) is shown in Table VII. As can be inferred from the table, genes regulated by AhR activation, including the drug metabolism CYP1A1 and CYP1A2 genes and the AhRR gene, were not differentially expressed to a statistically significant level when comparing the two groups. The CYP1A1 and CYP1A2 could not be quantified in the majority of samples, measurable

in just 38 (42%) and 27 (30%) subjects, respectively. However, the AhR and ARNT genes themselves showed significantly altered expression in the occupationally exposed group. Both were significantly upregulated 1.2-fold in the factory worker cohort compared to the population control group, after adjustment for the FDR (q-value <.05). Table VIII displays ΔCt values for AhR and ARNT gene expression by tertiles of total TEQ and suggests that those with the highest blood serum total TEQ concentrations had 1.4-fold higher expression of the AhR gene and 1.2-fold higher expression of the ARNT gene than those with the lowest total TEQ concentrations. These differences were statistically significant after adjustment for age, gender, and ethnicity.

TABLE VII

RELATIVE EXPRESSION OF SELECTED GENES RELATED TO THE ARYL HYDROCARBON RECEPTOR PATHWAY IN A DIOXIN-EXPOSED OCCUPATIONAL COHORT AND A POPULATION-SELECTED CONTROL GROUP

Gene	Study Group	n	mean ΔCt[a]	s.d.	Fold change[a]	p-value[b]	q-value[c]
AHR	Exposed	60	3.2	0.5	1.2	0.008	0.013
	Unexposed	30	3.5	0.5			
ARNT	Exposed	59	5.6	0.4	1.2	0.001	0.002
	Unexposed	30	5.9	0.4			
AHRR	Exposed	59	10.2	1.0	1.1	0.553	0.279
	Unexposed	29	10.4	1.2			
CYP1A1	Exposed	24	13.3	0.7	0.8	0.293	0.199
	Unexposed	14	13.0	0.9			
CYP1A2	Exposed	18	12.6	0.6	1.1	0.829	0.360
	Unexposed	9	12.7	1.0			

[a] Ct represents PCR cycle number at which DNA amount reaches a threshold value.
Housekeeping genes used in analysis: GAPDH, ACTB, GUSB, HPRT1, B2M, RPLPO.
ΔCt = Ct(gene of interest) - Ct(mean of housekeeping genes).
ΔΔCt = ΔCt(experimental) - Ct(control).
Fold change = $2^{(-\Delta\Delta Ct)}$.

[b] Welch-Satterthwaite t-test p-values shown.

[c] q-values (Storey method) used to account for the false discovery rate (FDR).

TABLE VIII

RELATIVE EXPRESSION OF THE AHR AND ARNT GENES BY TERTILE OF TOTAL TOXIC EQUIVALENTS (TEQ) CONCENTRATION

Gene	Total TEQ: Tertile[a]	n	mean ΔCt	s.d.	Fold change[b]	p-value[c]
AhR	≤ 38.6	30	3.4	0.5	-	<.001
	38.6–73.3	31	3.4	0.6	1.0	
	> 73.3	29	3.0	0.4	1.4	
ARNT	≤ 38.6	30	5.8	0.4	-	0.018
	38.6–73.3	31	5.6	0.5	1.2	
	> 73.3	28	5.6	0.3	1.2	

[a] TEQ measured in ppt, lipid-adjusted.

[b] Fold change = $2^{(-\Delta\Delta Ct)}$.

[c] p-values for trend from linear regression, adjusted for age, gender, and ethnicity.

2. **Effect of non-dioxin-like polychlorinated biphenyls on dioxin-induced aryl hydrocarbon receptor response**

Scatterplots of serum TEQ levels (ppt, lipid-adjusted) versus relative AhR and ARNT gene expression levels are depicted in Figures 7 and 8, respectively. Penalized B-splines were used to fit smoothed curves to the data (Eilers and Marx, 1996). Visual inspection suggests that relative expression of the AhR in the study samples seemed to increase (corresponding to a decrease in ΔCt) from serum levels of 0 to approximately 100 ppt TEQ, at which point expression levels first decreased and then leveled off. This pattern was not as clear for the relative expression of the ARNT gene. Further investigation into the modulating effects of NDL-PCB exposure on dioxin-induced AhR expression was carried out in an attempt to explain the apparent decrease in expression at higher serum levels of total TEQ. The effect of serum concentrations of several NDL-PCB congeners on relative AhR expression was investigated in those with serum dioxin levels (total TEQ) of greater than 100 ppt. Table IX shows mean ΔCt values for those with total TEQ blood levels of greater than 100 ppt, stratified by indicator NDL-PCB serum concentration tertiles. The results shown in Table IX demonstrate significantly decreased AhR

expression with increasing PCB 101 and PCB 153 blood levels in those with the highest total TEQ blood levels. Associations between AhR expression and serum levels of PCB 180 and PCB 28 were marginally significant.

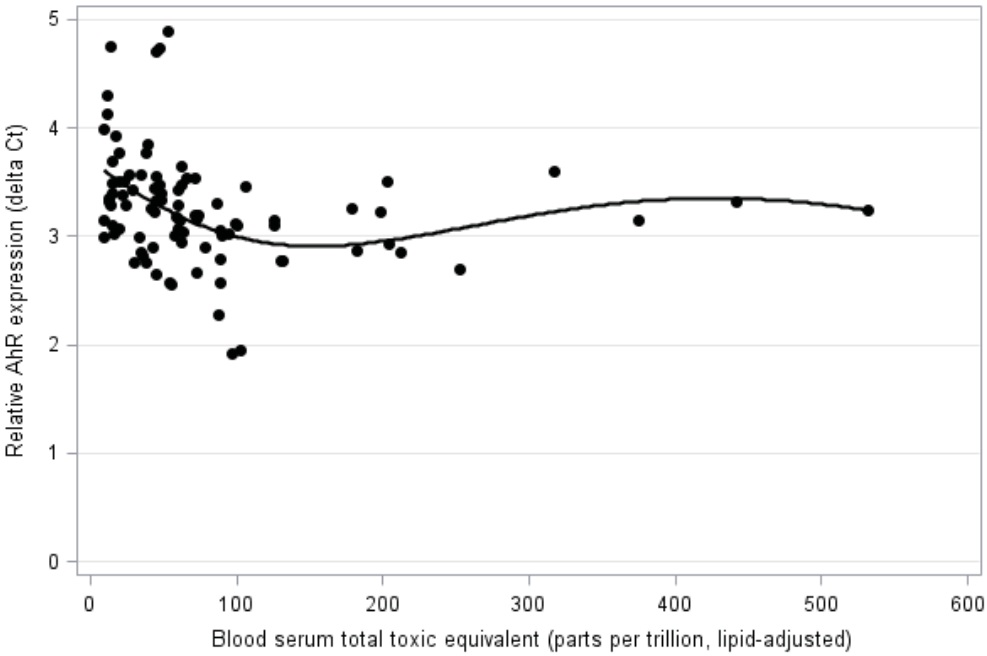

Figure 7. Scatterplot of relative AhR gene expression versus serum total toxic equivalency level.

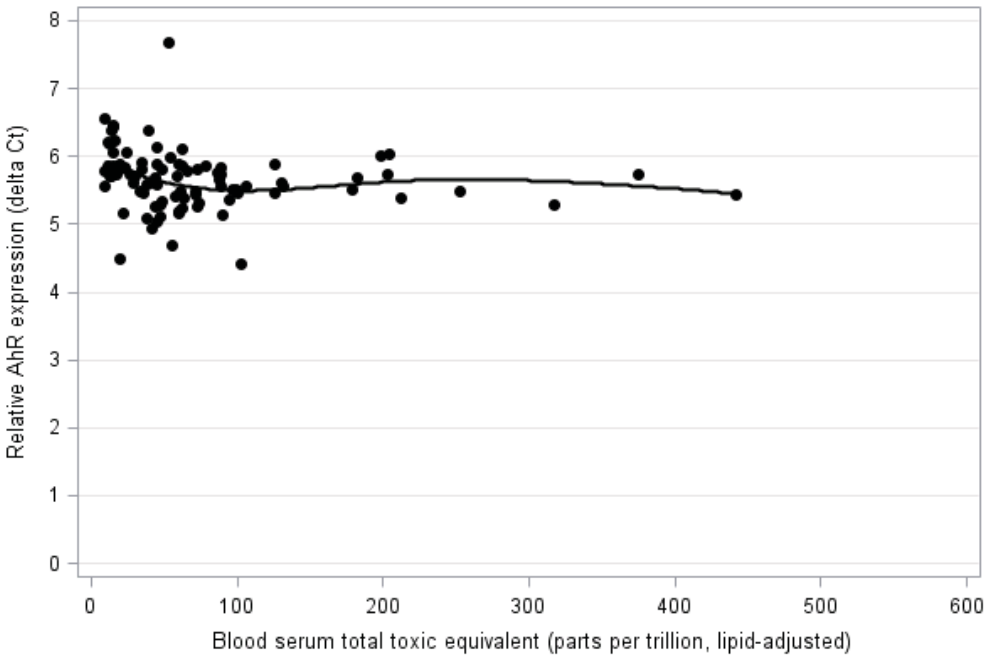

Figure 8. Scatterplot of relative ARNT gene expression versus serum total toxic equivalency level.

TABLE IX

EXPRESSION OF THE ARYL HYDROCARBON RECEPTOR GENE BY TERTILE OF NON-DIOXIN-LIKE PCB BLOOD CONCENTRATION FOR THOSE WITH >100 PPT TOTAL SERUM TEQ

NDL-PCB Tertile[a]		n	Mean ΔCt	s.d.	Fold change[b]	p-value[c]
PCB 28	≤1.8	5	2.83	0.57	ref	0.084
	1.8–3.6	6	3.08	0.21	0.84	
	>3.6	7	3.19	0.29	0.78	
PCB 52	≤.3	5	3.13	0.36	ref	0.660
	0.3–0.5	6	2.86	0.48	1.21	
	>0.5	7	3.17	0.27	0.97	
PCB 101	≤.5	5	2.87	0.56	ref	0.005
	0.5–1.0	8	3.06	0.27	0.87	
	>1.0	5	3.22	0.30	0.78	
PCB 118	≤28.0	9	3.18	0.30	ref	0.833
	28.0–49.0	4	2.78	0.61	1.32	
	>49.0	5	3.04	0.19	1.10	
PCB 138	≤40.4	3	3.11	0.34	ref	0.773
	40.4–69.3	6	2.93	0.57	1.14	
	>69.3	9	3.12	0.23	1.00	
PCB 153	≤57.6	2	2.37	0.58	ref	0.047
	57.6–91.4	6	3.10	0.28	0.60	
	>91.4	10	3.16	0.27	0.58	
PCB 180	≤24.3	2	2.37	0.58	ref	0.064
	24.3–34.5	4	3.13	0.32	0.59	
	>34.5	12	3.14	0.26	0.58	

[a] PCB serum levels measured in ppb, lipid-adjusted.

[b] Fold change = $2^{(-\Delta\Delta Ct)}$.

[c] p-values for trend from linear regression, adjusted for age, gender, and ethnicity.

To further assess the modifying role of PCB 153 and PCB 180, the NDL-PCBs found to be most abundant in the blood samples from the study population, on the effect of dioxin exposure on AhR and ARNT gene expression, multivariable linear regression models were fit separately for those with high versus low PCB levels (Table X). High levels of PCB 153 were defined as those exceeding the 90th percentile of serum PCB 153 concentrations measured in the US NHANES study population, or 71.8 ppb.

Because only a small number of subjects in our study exceeded the 90th percentile of serum PCB 180 concentrations measured in the US NHANES study population, high levels of PCB 180 were defined as those exceeding the 75th percentile, or 37.1 ppb.

TABLE X

MULTIVARIABLE LINEAR REGRESSION RESULTS: FOLD CHANGE IN AHR AND ARNT EXPRESSION IN SUBJECTS WITH HIGH AND LOW PCB 153 AND PCB 180 CONCENTRATIONS

	AhR		ARNT	
NDL-PCB[a]	Fold Change[b]	95% CI	Fold Change[b]	95% CI
PCB 153[c]				
>71.8	1.00	0.99–1.02	1.00	0.99–1.01
≤71.8	1.04	1.01–1.07	1.03	1.00–1.06
PCB 180[d]				
>37.1	1.00	0.99–1.01	1.00	0.99–1.01
≤37.1	1.02	1.00–1.04	1.01	0.99–1.02

[a] Serum NDL-PCB levels measured in ppb, lipid-adjusted.

[b] Fold change = $2^{(-\Delta\Delta Ct)}$; estimates represent the fold change for each 10-unit increase in serum TEQ, ppt, lipid-adjusted; all models adjusted for age, gender, ethnicity, and current smoking status.

[c] 71.8 ppb represents the 90th percentile of serum values for PCB 153 in the NHANES population.

[d] 37.1 ppb represents the 75th percentile of serum values for PCB 180 in the NHANES population.

Though this analysis did not have the statistical power to evaluate the influence of smoking on the interaction between NDL-PCBs and total dioxin TEQ, the effect of smoking on the expression of the genes of interest is presented in Table XI. As shown in the table, of the gene considered in this analysis, only the AhRR gene was differentially expressed in current smokers, as compared to former smokers and nonsmokers. Specifically, the expression of AhRR in current smokers was 1.74 times higher.

TABLE XI

FOLD CHANGE IN AHR, ARNT, AHRR, CYP1A1, AND CYP1A2 EXPRESSION FOR CURRENT SMOKERS COMPARED TO FORMER AND NEVER SMOKERS

	Current Smokers[a]		
Gene	n	Fold Change[b]	95% CI
AhR	89	1.22	0.95–1.58
ARNT	88	1.15	0.92–1.43
AhRR	87	1.74	1.04–2.91
CYP1A1	37	0.97	0.61–1.56
CYP1A2	27	0.98	0.49–1.94

[a] There were 10 current smokers in the sample.

[b] Fold change = $2^{(-\Delta\Delta Ct)}$.

D. **Discussion**

Our analysis demonstrated a significant association between total dioxin concentration (total TEQ) in PBMCs and expression of the AhR and the ARNT genes in subjects from two Eastern European cohorts, one occupationally exposed and one environmentally exposed to dioxins. Surprisingly, no such differential expression of several genes regulated by the AhR (including CYP1A1, CYP1A2, and AhRR) was detected. The AhR pathway has been well established as a mediator of the toxic response to dioxins (White and Birnbaum, 2010). Activation of the receptor leads to the transcription of genes encoding drug metabolizing enzymes and inhibitory proteins, among others (Safe, 1989; Nagao, 1990).

Unexpectedly, we observed increased expression of the AhR and the ARNT genes without similar changes in downstream mRNA levels. In analyses of peripheral blood lymphocyte samples from residents of Seveso, Italy, 20 years after an industrial accident contaminated the city, AhR transcript levels were found to be negatively correlated to current plasma TCDD levels (Landi et al., 2003). These findings are supported by cell culture and animal studies that suggest that the AhR protein is down-

regulated following ligand exposure. Some speculate that the purpose of AhR proteolysis and down-regulation following activation is to reduce the response to stable ligands (such as aryl hydrocarbons) which are not readily metabolized or to conserve the availability of the ARNT, which is involved in numerous other signaling pathways (Pollenz, 2002).

However, the increased expression of the AhR in our study may be a result of more sustained (chronic) exposure to dioxins, as compared with the high acute exposure experienced by the residents of Seveso. The down-regulation of the AhR discussed previously typically occurs after high doses or when measurements are taken soon after TCDD administration (Harper et al., 2006). Persistent, lower-dose exposure to dioxins may have a different effect on AhR expression, as reported in studies that found either stable or increased AhR levels in hepatic cells (Sloop and Lucier, 1987; Franc et al., 2001).

The patterns of gene expression detected in our study might also be explained by the type of tissue examined. Constitutive AhR expression can vary substantially among tissue types. Expression of AhR in lymphocytes (e.g., macrophages, B-cells, T-cells) seems to increase upon activation, and dioxins have been shown to activate these cells (Kerkvliet et al., 2002; Allan and Sherr, 2005). One research team reported a similarly increased AhR expression in murine T-cells upon activation, but could not detect DNA binding (and subsequent CYP1A1 production) even after treatment with TCDD (Lawrence et al., 1996). Furthermore, there may be sizable inter-individual variability in the expression of CYP1A1 in human lymphocytes, which may limit their use as biomarkers of CYP1A1 expression (van Duursen et al., 2005).

Whether our observed changes in AhR expression are primarily a result of direct or indirect effects of receptor activation is debatable. For instance, proinflammatory conditions brought on by AhR-mediated transcription can augment AhR expression (Champion et al., 2013). However, there are many variables that regulate AhR expression other than chemical exposure, including hormonal factors, age and developmental stage, growth factors, cytokines, cell density, cell differentiation, and neoplastic

transformation (Harper et al., 2006). Making sense of the complexities involved in AhR expression and regulation remains a challenge.

Our results additionally suggest an interaction between dioxin and NDL-PCB serum levels on AhR expression. As illustrated in Figure VII, relative AhR expression appeared to generally increase with total TEQ levels until approximately 100 ppt, at which point average expression increased and leveled off. This pattern was not as obvious with ARNT expression (Figure 8). Among those subjects with serum TEQ levels of greater than 100 ppt, PCB 153 was found to be inversely associated with AhR gene expression (Table IX). The association with PCB 180 was marginally significant. Multivariable linear regression results for those with high and low levels of these two NDL-PCBs provided further support of an interaction between NDL-PCBs and total dioxin TEQ. Specifically, the models suggested that the observed association between serum TEQ and AhR/ARNT expression more or less disappeared with concurrently high PCB 153 or PCB 180 blood concentrations. This attenuation was most dramatic for AhR expression in those with high PCB 153 levels.

The antagonistic effects of specific NDL-PCB congeners on dioxin-induced gene expression and enzyme induction are documented in several cell culture studies, with the antagonistic potential of PCB 153 specifically highlighted in most (Bannister et al., 1987; Haake et al., 1987; Biegel et al., 1989; Davis and Safe, 1989). A few have further demonstrated that several NDL-PCBs, including PCB 153, diminish the expression of AhR-mediated CYP1A1 with concurrent exposure to dioxins by preventing the initiation of DNA transcription in the nucleus (Chen and Bunce, 2004; Chubb et al., 2004). Based on their findings, Suh et al. (2003) concluded that di-ortho-substituted PCBs (which include PCB 153) interfere with the dioxin-induced activation of the cytosolic AhR complex to antagonize CYP1A1 induction in a murine cell line of B-lymphocytes. These antagonistic properties may have also contributed to the patterns of AhR-mediated gene expression (e.g., CYP1A1, CYP1A2, AhRR expression) observed in our study.

The antagonistic effects of PCB 153 on AhR expression suggested by our findings open several interesting avenues for future investigation. For one, the complex interplay between various pathways involved in dioxin- and NDL-PCB-mediated toxicity is still poorly understood. The NDL-PCBs, such as PCB 153, have been identified as ligands for the pregnane X receptor and the constitutive androstane receptor, both of which regulate the expression of genes related to xenobiotic metabolism and elimination (Waxman, 1999). There is substantial crosstalk between these nuclear receptors and the AhR, given their common role in xenobiotic metabolism (Pascussi et al., 2008). The pregnane X receptor may play a role in the regulation of AhR expression, as demonstrated in human hepatocytes (Maglich et al., 2002). Future studies aimed at characterizing how these pathways interact with chronic exposure to dioxins and NDL-PCBs would aid in our understanding of the mechanisms through which mixtures of chemicals exert their toxicity in human populations.

1. **Limitations**

Our study results should be interpreted with caution, given the relatively small sample size. This is particularly relevant to our findings regarding the interaction between dioxins and NDL-PCBs on AhR gene expression. Further, we did not have the statistical power to make valid conclusions following stratification of our results by potentially influential factors, such as smoking status. We observed statistically increased expression of the AhRR gene in the ten current smokers, as compared to former smoker and nonsmokers, in our study sample. This finding suggests that constituents in cigarette smoke may activate the AhR in humans, resulting in the upregulation of downstream genes, including the AhRR. Given the wide array of chemicals found in cigarette smoke, future study of the relationship between chronic dioxin and NDL-PCB exposure by smoking status could prove fruitful.

Additionally, any conclusions drawn regarding the independent effects of individual NDL-PCB congeners may be prone to error, given the relatively high correlations among these analytes. Those

with higher serum levels of NDL-PCBs tended to have relatively high levels of more than one congener. However, as discussed previously, our results are supported by several animal and in-vitro studies, and thus represent biologically plausible findings.

The degree to which our results are generalizable to other tissue types may be limited. The expression of the AhR and the mechanisms by which the AhR is regulated differ among cell types. Furthermore, PBMCs represent a heterogeneous mix of cells, which include neutrophils, basophils, eosinophils, and lymphocytes. Differing proportions of each cell type among study samples can affect the interpretation of gene expression data (Hamadeh and Afshari, 2004). Despite these limitations, given that the immune system is a target of dioxin toxicity, and since peripheral blood lymphocytes are relatively easy to collect, it seems prudent to study the effects of chemical exposures on the functioning of these cells.

2. **Conclusions**

In this study of two Eastern European cohorts with measurable and varying levels of dioxin and NDL-PCB exposure, we observed increasing AhR and ARNT gene expression with increasing dioxin levels (measured as total TEQ—ppt, lipid-adjusted) without differential expression of genes known to be regulated by the AhR (CYP1A1, CYP1A2, AhRR). We also identified NDL-PCB congeners, chiefly PCB 153, that may modify the relationship between serum dioxin levels and AhR expression in a human population. To our knowledge, the antagonistic effect of NDL-PCBs on dioxin-induced gene expression has not been reported using blood samples from a healthy human cohort. The complex exposures experienced by humans and the uncertainties inherent to the extrapolation of results from animal and in-vitro models necessitate the need to study molecular effects using population-based studies. Our results support previous findings and encourage the testing of new hypotheses regarding dioxin and NDL-PCB toxicity.

V. RISK FACTORS ASSOCIATED WITH CHLORACNE DEVELOPMENT IN A COHORT OCCUPATIONALLY EXPOSED TO 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN

A. Background

Dioxins are a class of chemical contaminants that have become ubiquitous in the environment, primarily as a result of industrial processes (ATSDR, 1998). Their hydrophobicity and vapor pressure make them highly persistent and allow them to exist in the atmospheric, terrestrial, or aquatic environmental compartments (Aberg et al., 2008; Mackay et al., 2006). Human exposure to dioxins is believed to occur through the oral, dermal, and inhalation routes (ATSDR, 1998). Included in this group of substances are the PCDDs, the PCDFs, and a subcategory of DL-PCBs that share the same mode of action (IARC, 1997). The most toxic (and most well-studied) member of this group is TCDD (ATSDR, 1998).

Health effects that have been linked to dioxin exposure are numerous and include cancer, immune deficiency, reproductive/developmental abnormalities, nervous system disorders, endocrine disruption, and pulmonary disease (Schecter et al., 2006). Dermal effects have also been documented. In fact, chloracne is one of the few clinical findings in humans consistently associated with such an exposure (IOM, 2009). As such, chloracne has been the subject of numerous studies of cohorts exposed to dioxins.

Clinically, chloracne cases present with follicular hyperkeratosis (comedones) that may or may not occur with further inflammatory nodules and noninflammatory straw-colored cysts. Any part of the body can be affected, though chloracne is usually observed on the face, neck, and genitals. It can develop relatively soon after exposure to chloracnegens—usually within a few weeks—and may clear up in as short as several months after exposure ceases or as long as 30 or more years after initial onset

(ATSDR, 1998; Ju et al., 2012). Histopathology includes miniature-to-absent sebaceous glands, hyperplasia of the epidermis, and hyperpigmentation of the stratum corneum (Ju et al., 2012).

Though development of chloracne is indicative of very high exposure to chloracnegens, very high exposure to dioxins does not guarantee chloracne development (Beck et al., 1989). This finding is supported by studies of residents with documented exposure to TCDD after the Seveso, Italy, accident (Baccarelli et al., 2005). Attempts have been made to quantify the blood levels necessary to develop chloracne (Coenraads et al., 1999; Neuberger et al., 1991), though the dissimilarities in study findings suggest that there may not necessarily be a correlation between serum dioxin concentration and the occurrence of chloracne (IOM, 2009). Case studies also indicate individual variations in disease severity (Geusau et al., 2001), suggesting that there may be factors other than exposure level that predict how serious symptoms will be.

Evidence points to the AhR signaling pathway as a mediator of most of the effects of TCDD and other dioxin-like congeners. It follows that there are several studies that implicate the AhR pathway in chloracne pathogenesis (Tauchi et al., 2005; Tang et al., 2008; Ju et al., 2011). Expression of the AhR has been demonstrated in human keratinocytes, skin fibroblasts, sebaceous glands, and immortalized SZ95 sebocytes (Bock and Köhle, 2009; Ju et al., 2011). Overexpression of the AhR is inhibited by the AhRR protein, which competes with the AhR to form a heterodimer with the ARNT protein in a negative feedback loop (Mimura et al., 1999). In 2004, Ukrainian presidential candidate Victor Yushchenko consumed a meal poisoned with TCDD. Gene expression analysis of his skin cells revealed the induction of the cytochrome P450 (CYP) 1A1, CYP1A2, and CYP1B1 genes (which are involved in drug metabolism), as well as the AhRR gene (Saurat et al., 2012).

The major aim of the analysis presented here was to identify factors that might explain why only some individuals who are exposed to high concentrations of dioxins develop chloracne. This study focused on a cohort of chemical factory workers who were occupationally exposed to dioxins.

Demographic, occupational, environmental, behavioral, and physiological factors were obtained from surveys, physical examinations, and blood samples to identify possible risk factors for chloracne development. Additionally, we hypothesized that the AhRR would be expressed to a higher degree in the PBMCs of those who did not develop the disease despite sizable dioxin exposure. The results may have implications for risk assessment in human populations and provide information useful to the future investigation of individual susceptibility to chloracne development.

B. Methods

1. Study participants

In 2009, 218 former workers with historical occupational exposure to dioxins while engaged in the manufacture of chlorinated phenols and their phenoxy-acid esters were recruited. The chemical plant where these subjects worked, located in Eastern Europe, was active from the early 1940s until 2004 and produced various chlorinated chemicals, from household bleach to pesticides. Emissions discharged by toxic waste incinerators at the plant contaminated the immediate workspace and the surrounding city with dioxins.

The factory workers included in this study were involved in the production of TCP and TCP-Cu, 2,4,5-T, and 2,4-D. The potential for high acute exposure to dioxins presumably varied by department. The manufacture of lower chlorinated phenols at the plant began in 1960. Trichlorophenol and TCP-Cu were produced in one department of the plant. The annual production capacity was 120 tons of TCP and 35 tons of TCP-Cu between 1961 and 1974 and 1,000 tons of TCP and 100 tons of TCP-Cu between 1975 and 1987. In 1987, the manufacturing of TCP and TCP-Cu was discontinued. Samples of technical 2,4,5-TCP contained up to 0.65 mg/kg of TCDD.

The experimental production of the N-butyl esters of 2, 4, 5-T began in November 1964 in another department. The industrial manufacturing of this product began in May 1965 and was

discontinued in 1968. The production capacity of 2,4,5-T was approximately 50–60 tons per month. A series of accidents in this department resulted in contamination of the plant and potentially high occupational exposure of plant workers. During one of these accidents, approximately 8 tons of 2,4,5-T spilled onto the floor and subsequently traveled into the disposal system. Industrial hygiene controls were not adequate and worker overexposures occurred on numerous occasions (source: personal communication with plant workers).

The experimental manufacturing of the esters of 2,4-D at the plant started in the early 1960s. From 1963 through 1989, esters of 2,4-D (filtration scheme) were produced. From 1986, the production of 2,4-D (extraction scheme) occurred in another department. In 1990, the plant commenced production of the sodium salt of the monochloroacetic acid of 2,4-D using modern technology. Metaxone (2-Methyl-4-chlorophenoxyacetic acid) was produced at the plant from 1966 through 1989.

Detailed information regarding each worker's occupation at the plant was obtained from plant personnel records and a survey completed by each study subject. Surveys were administered to collect demographic (e.g., age, ethnicity), behavioral (e.g., smoking status), and medical (e.g., previous diagnoses, family history of cancer) information from each worker and control subject. Qualified professionals performed physical examinations and collected blood samples for serum dioxin and biomarker studies. The subset of medical and occupational history data that were related to study hypotheses were entered into a database by the UIC Data Management Center in Kyiv, Ukraine, and subsequently transferred to the UIC. After they were drawn, blood samples from the 218 exposed and 150 unexposed participants were immediately cryopreserved at -80°C and transferred in dry ice to partner laboratories at the CDC, Griefswald University in Germany, and the University of Milan in Italy. This study was approved by the CDC NIOSH and the UIC Institutional Review Boards.

2. **Exposure assessment**

The CDC analyzed a 50 mL archived serum sample from each study participant for 7 PCDDs, 10 PCDFs, 4 coplanar PCBs (c-PCBs, including PCB 77, 81, 126, and 169), and 38 ortho-substituted PCBs by high-resolution gas chromatography/isotope-dilution high-resolution mass spectrometry (HRGC/ID-HRMS) Method 28 (Patterson et al., 1987). All dioxin analyses were conducted at the same testing facility with the same assay, equipment, and standardized procedures. Total lipid content of each specimen was estimated using a summation method (Akins et al., 1989), and analytical results were reported on a lipid-adjusted basis as pg/g lipid ppt for PCDDs, PCDFs, and c-PCBs, and as ng/g lipid ppb for ortho-substituted PCBs. Congener-specific detection limits were corrected for sample weight and analyte recovery. For individual analytes below the LOD, a value of zero was assigned. Total toxic equivalents were calculated according to the WHO 2005 relative toxic potency ranking scheme (Van den Berg et al., 2006). The geometric mean (and 95% CIs) serum levels for two dioxins (TCDD, PCB 126) and two representative NDL-PCBs (PCB 153, PCB 180) in this cohort were compared to serum levels reported for 2003–2004 in the general US population (NHANES).

Because serum concentration of dioxins was measured, on average, close to 20 years since subjects last worked at the chemical plant, we attempted to calculate other estimates of exposure that might more accurately characterize body burden at the time of employment. This is especially relevant for the outcome of chloracne, since this condition would likely have developed within weeks of exposure (ATSDR, 1998). For one alternate exposure measure, we back-extrapolated serum TCDD levels to the last day of employment for each worker using the half-life for TCDD as estimated by calculating the diminution rate in the cohort, based on at least two blood measurements for each individual. This diminution rate corresponded to a half-life of 7.8 years, which was the same as that estimated for adults living in Seveso, Italy, the site of a 1976 industrial accident that contaminated the surrounding areas

with dioxin (Needham et al., 1994). Back-extrapolated serum levels (ppt) were calculated using Equation 1 (Checkoway et al., 2004).

$$C_t = C_o e^{-kt} \quad (1)$$

Where:

C_t = serum concentration at time of sample collection (ppt)

C_o = peak concentration, last day of employment (ppt)

k = rate constant ($0.693/t_{1/2}$), where $t_{1/2}$ = 7.8 years

t = time from last day of employment to sample collection (years)

An estimate of relative cumulative exposure was also calculated for this study. In addition to current blood serum dioxin concentrations, detailed information regarding job title and department for each worker was made available, though no area or personal sampling data from the plant could be obtained. Therefore, correlations between dioxin air/dermal/ingestion exposures and dioxin blood concentration were unknown and could not be feasibly determined (Esmen, 1981). Without sampling data, the exposure reconstruction could only be expressed in terms of a unitless relative exposure parameter. This parameter was such that if a person was exposed to dioxin at levels that corresponded to this parameter, then current blood dioxin levels would be equivalent to the measured levels. Ultimately, each job title was assigned a categorical value as a proxy for exposure concentration. Cumulative exposure was broadly estimated using Equation 2, which takes into account concentration, job duration, and the metabolism/excretion of dioxin between exposures (Phillips and Esmen, 2007).

$$\text{Cumulative Exposure} = \sum C_i * \Theta_{ij} \exp[-\alpha t_{ij}] \quad (2)$$

Where:

C_i = relative dioxin concentration in job i

Θ_{ij} = time spent by worker j in job i

α = rate at which blood dioxin concentration diminishes over time

t_{ij} = time spent at the factory minus time spent in job i for worker j

Finally, we considered a metric that might allow for the delineation of workers with potentially high acute dioxin exposures. A series of accidents at one of the departments in the chemical plant occurring between 1964 and 1968 resulted in contamination of the plant and surrounding territory, along with larger than usual occupational exposures. During one of these accidents, approximately 8 tons of 2,4,5-T were spilled on the floor, subsequently traveling into the disposal system. According to the plant workers, industrial hygiene controls were not adequate and worker overexposures occurred on numerous occasions. We investigated the distribution of chloracne cases by job department to determine whether work in any particular area of the plant might represent an independent risk factor for disease development. In addition, we created a binary variable to indicate whether each worker was present in the factory during the time period when the industrial accidents occurred. Taken together, these variables might reasonably represent high acute exposure to dioxins as a result of industrial contamination.

3. **Outcome assessment**

For the data analysis, the outcome of interest was a clinical diagnosis of current or past chloracne, as ascertained by a dermatologist. Diagnosis was based on a review of past medical records, questions regarding medical history, and current physical examination.

4. **Gene expression analysis**

The PAXgene Blood RNA Kit was used to isolate total RNA (Qiagen-PreAnalytix, Hombrechtikon, Switzerland) in a subset of the worker cohort (n=60). To obtain the random sample, subjects with serum TCDD levels below 10 ppt were excluded. The remaining participants were grouped by tertile of serum TCDD level, and 20 from each tertile were randomly selected for gene expression analysis. Qiagen prepared a customized array of 83 genes selected by our team for this study and all future studies of gene expression in the cohort. These candidate genes were chosen based on several criteria: (1) they were known to be key components of the AhR, human lymphoma, and apoptosis pathways; (2) they were shown to be upregulated greater than 1.30-fold or down-regulated at least 0.65-fold in previous studies of dioxin-exposed humans; or (3) their expression was shown to be changed 5-fold in previous studies of dioxin-exposed human cell lines. Complementary DNA (cDNA) was synthesized with a RT2 First Strand kit (SABiosciences, Frederick, Maryland, USA) and analyzed with specific RT2 Profiler PCR arrays by RT-PCR using Syber Green chemistry. The PCR array is a complete system for pathway-focused gene expression analysis with some components that guarantee high-quality, reproducible, and reliable gene expression data. The Profiler PCR array includes 89 RT2 qPCR primer assays (including 5 housekeeping genes) and a proprietary control panel to investigate contamination with genomic DNA (gDNA), quality of the reverse transcription reaction, and real-time efficiency (Qiagen Inc., Mississauga, Canada). All RT-PCR runs were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, California, USA).

5. **Statistical analysis**

After assessment of the univariate distributions of the outcome and exposure variables of interest in this analysis, bivariate relationships between environmental exposure and outcome variables were investigated using contingency tables and χ^2 tests of association. Dioxin exposure

measures were $\log_{10}$-transformed to account for their skewed distributions. Statistical models were fit to the data to obtain a least-biased estimate of the associations between exposures of interest and chloracne development. Multivariable logistic regression models with model-based standardization were applied to calculate RR estimates (Localio et al., 2007) for each exposure measure, and 95% Cis were obtained using a bias-corrected bootstrap method (Mooney and Duval, 1993). A backward elimination strategy was considered for inclusion in the final risk model, and product terms were included to assess interactions between dioxin exposure measures and various other factors (Kleinbaum et al., 2007). A priori knowledge was also considered for inclusion in the final model. Likelihood ratio tests were employed to compare nested models (Rothman et al., 2008) and identify significant risk factors.

The subsample of gene expression data for the exposed group (n=60) was used to assess differential AhR, ARNT, and AhRR gene expression between those who developed chloracne and those who did not. Relative gene expression levels were determined using the ΔΔCt method (Livak and Schmittgen, 2001). Crude statistical comparisons by risk group were performed, adjusting for age, gender, and ethnicity and accounting for the FDR using QVALUE software written by Alan Dabney and John Storey (Storey, 2002). Relative expression levels were incorporated into the logistic regression models described above, modeled to the data for the subsample, to determine whether any excess variation could be explained by these expression levels.

All statistical analyses were performed using SAS 9.3 (Cary, North Carolina, USA), Stata 12.1 (StataCorp, College Station, Texas), or QVALUE.

C. **Results**

The distribution of selected demographic and physiological factors in the worker cohort, stratified by chloracne status, is shown in Table XII. Of 210 workers who completed the dermatologist

examination, 45 were diagnosed with present or past chloracne. The crude association between male gender and chloracne development was statistically significant (RR=3.1, 95%CI: 1.6–6.0). Risk of chloracne development was also statistically increased in both former smokers (RR=2.9, 95%CI: 1.5–5.8) and current smokers (RR=4.2, 95% CI: 2.3–7.6). The significant gender effect was likely confounded by smoking status, as 83 of 84 (99%) current/former smokers in the worker cohort were male, and never-smoker females were just as likely to have been diagnosed with chloracne as never-smoker males (data not shown). Borderline significant associations with chloracne development were estimated for ethnicity, age at recruitment, and BMI. Serum lipid, cholesterol, and triglyceride levels were similar among workers with a history of chloracne and those without. Those with a history of chloracne were more likely to have a current diagnosis of hypertension (RR=1.8, 95% CI: 1.0–3.3). None of the other medical or occupational survey items were found to be significantly associated with chloracne diagnosis (data not shown). None of the workers reported a personal or family history of lymphoma, while one worker had a parent who was diagnosed with leukemia.

TABLE XII

CRUDE ASSOCIATIONS BETWEEN SELECTED FACTORS AND CHLORACNE STATUS

	Chloracne[a]				
	Yes		No		
	#	%	#	%	RR (95%CI)
Gender					
Male	36	30.3	83	69.8	3.1 (1.6–6.0)
Female	9	9.9	82	90.1	1.00 (ref)
Ethnicity					
Asian	30	24.4	93	75.6	1.8 (0.9–3.4)
Other	5	35.7	9	64.3	2.6 (1.1–6.5)
Caucasian	10	13.7	63	86.3	1.00 (ref)
Smoking Status					
Current	19	45.2	23	54.8	4.2 (2.3–7.6)
Former	12	31.6	26	68.4	2.9 (1.5–5.8)
Never	14	10.8	116	89.2	1.00 (ref)
Hypertension[d]					
Yes	34	25.8	98	74.2	1.8 (1.0–3.3)
No	11	14.3	66	85.7	1.00 (ref)
	n	Mean (Median)	n	Mean (Median)	p-value[b]
Age at recruitment	45	68.7 (70.0)	165	67.1 (69.0)	0.076[c]
Age at plant hire	44	23.6 (23.0)	165	24.6 (23.1)	0.234[c]
BMI[d]	41	27.0 (25.9)	155	28.4 (27.7)	0.077
Lipids[d]	45	728.0 (711.1)	165	724.5 (693.6)	0.897
Cholesterol[d]	45	227.3 (221.0)	165	224.9 (222.0)	0.750
Triglycerides[d]	45	149.7 (138.0)	165	151.7 (128.0)	0.886

[a] Includes all cohort subjects with known chloracne status (n=210).

[b] Independent samples student's t-test used to obtain p-value.

[c] Satterwaite method used to test significance of t-test due to unequal variance.

[d] Based on measurements taken after study recruitment.

The proportion of employees who developed chloracne, along with average current serum TCDD levels, in each area of the plant is displayed in Table XIII. Among those who ever worked in the production of 2,4,5-T, about 30% developed chloracne, compared to about 10% among those who

worked in the manufacture of TCP/TCP-Cu, and about 22% among those who ever worked in 2,4-D production. Four of the 20 (20%) subjects who ever worked in the plant's central laboratory developed chloracne, while two of four (50%) of those who ever worked in the accident prevention or rescue departments and one of two (50%) of those who ever worked in the maintenance department additionally developed the skin condition. Of the workers employed in other departments, about 19% developed chloracne, though variance in current serum dioxin levels and uncertainty regarding operations in these departments suggests a potentially large heterogeneity in exposure. However, many of the workers worked in multiple departments over the course of their tenure at the plant. When considering those subjects who only ever worked in one area of the factory, the proportion of chloracne cases was highest in the 2,4,5-T production department (~38%) and lower in the 2,4-D production departments (~15%) and in departments categorized as "other" (~11%). None of the subjects worked exclusively in the chlorobenzene/metaxon production department, the central laboratory, the accident prevention and rescue departments, or the maintenance department.

The highest mean/median current serum TCDD levels (ppt) were observed in subjects who worked in the TCP/TCP-Cu production department; these levels were approximately 10 to 20 times higher than those of any other department assessed. Mean and median TCDD levels were generally lower in those who developed chloracne versus those who did not in the other departments.

TABLE XIII

PROPORTION OF WORKERS DEVELOPING CHLORACNE BY DEPARTMENT WORKED

	Chloracne[a]							
	Yes				No			
			Serum TCDD (ppt[b])				Serum TCDD (ppt[b])	
	%	n	mean	median	%	n	mean	median
2,4,5-T Production								
Ever worked	29.6	21	53.8	17.1	70.4	50	92.0	31.5
Only worked	37.5	6	26.7	16.7	62.5	10	24.9	23.8
TCP, TCP-Cu Production								
Ever worked	9.5	4	232.5	215.5	90.5	38	146.8	100.9
Only worked	14.3	1	272.0	272.0	85.7	6	144.4	100.9
Chlorbenzene, Metaxon Production								
Ever worked	30.0	3	15.8	10.8	70.0	7	28.9	33.5
Only worked	-	0	-	-	-	0	-	-
2,4-D Production								
Ever worked	22.1	25	20.8	12.7	77.9	88	32.7	21.5
Only worked	15.4	4	22.5	22.4	84.6	22	20.3	19.1
Central Lab								
Ever worked	20.0	4	36.5	33.9	80.0	16	29.0	22.1
Only worked	0.0	0	-	-	100.0	2	29.1	29.1
Accident Prevention/Rescue								
Ever worked	50.0	2	14.4	14.4	50.0	2	19.3	19.3
Only worked	-	0	-	-	-	0	-	-
Maintenance								
Ever worked	50.0	1	4.6	4.6	50.0	1	16.8	16.8
Only worked	-	0	-	-	-	0	-	-
Other departments[c]								
Ever worked	19.2	23	25.6	19.1	80.8	97	36.0	22.9
Only worked	11.1	2	22.4	22.4	88.9	16	23.4	19.7
All Workers	21.4	45	40.5	16.2	78.6	165	53.8	23.5

[a] Includes all cohort subjects with known chloracne status (n=210).

[b] ppt = parts per trillion.

[c] Includes those who did not work in the aforementioned areas of the plant.

Presence in the factory between the years of 1964 and 1968, a period during which several industrial accidents occurred, was assessed independently as an estimate of acute dioxin exposure. Relative risks and 95% CIs for each exposure variable, obtained from logistic regression with model-based standardization, are listed in Table XIV. Estimates of blood serum TCDD levels and cumulative exposure were not statistically associated with the diagnosis of chloracne. However, working at the plant during the time period 1964–1968 was found to be statistically associated with chloracne diagnosis even after adjustment for demographic factors and smoking status as reported at the time of recruitment.

TABLE XIV

LOGISTIC REGRESSION MODELS OF THE RELATIONSHIP BETWEEN CHLORACNE STATUS AND EXPOSURE MEASURES

	Crude Analysis		Adjusted Analysis[a]	
Exposure	RR[b]	95% CI[c]	RR[b]	95% CI[c]
Current TCDD levels[d]	0.57	(0.39–0.95)	0.78	(0.50–1.30)
Back-extrapolated TCDD levels[d]	0.71	(0.61–1.01)	0.86	(0.66–1.32)
Relative cumulative exposure[d]	1.20	(0.77–2.63)	0.89	(0.71–1.69)
Present 1964–1968	2.39	(1.21–6.07)	2.64	(1.16–8.77)

[a] Adjusted for age at hire, gender, ethnicity, and smoking status.

[b] Relative risk estimated from logistic regression model using model-based marginal standardization technique, as described by Localio et al., 2007.

[c] Estimated using bias-corrected bootstrap method described by Mooney and Duval, 1993.

[d] Log10-transformed.

Of the various demographic, occupational, environmental, behavioral, and physiological factors assessed, smoking status showed the strongest association with chloracne development in this

occupational cohort of chemical factory workers (Table XV). The risk of chloracne development was elevated in both former smokers (RR=2.80, 95%CI: 1.04–6.95) and current smokers (RR=4.16, 95%CI: 2.08–10.15).

TABLE XV

LOGISTIC REGRESSION MODELS OF THE RELATIONSHIP BETWEEN CHLORACNE STATUS AND SMOKING STATUS

Tobacco Use[a]	Risk Ratio[b]	(95% CI)[c]
All workers		
Current smoker	4.16	(2.08–10.15)
Former smoker	2.80	(1.04–6.95)
Never smoker	1.00	-

[a] Models adjusted for age at hire, gender, ethnicity and whether employed between 1964 and 1968; Includes subjects with known dates of employment and chloracne status (n=209).

[b] Estimated from logistic regression model using model-based marginal standardization technique, as described by Localio et al., 2007.

[c] Estimated using bias-corrected bootstrap method described by Mooney and Duval, 1993.

As inferred from the previous tables and statistical models, measures of serum TCDD in the cohort as a whole were lower, on average, in those with chloracne versus those without chloracne. The relationship between serum TCDD and chloracne development is stratified by smoking status (former/current- versus never-smokers) in Figure 9. Among never-smokers, mean and median serum TCDD levels were slightly higher among those who developed chloracne, compared to those who did not develop the skin condition. Table XVI shows that current smokers had a higher average pack-year

experience than did former smokers, and the geometric mean serum TCDD and TEQ levels were lowest among current smokers in both the chloracne and non-chloracne groups.

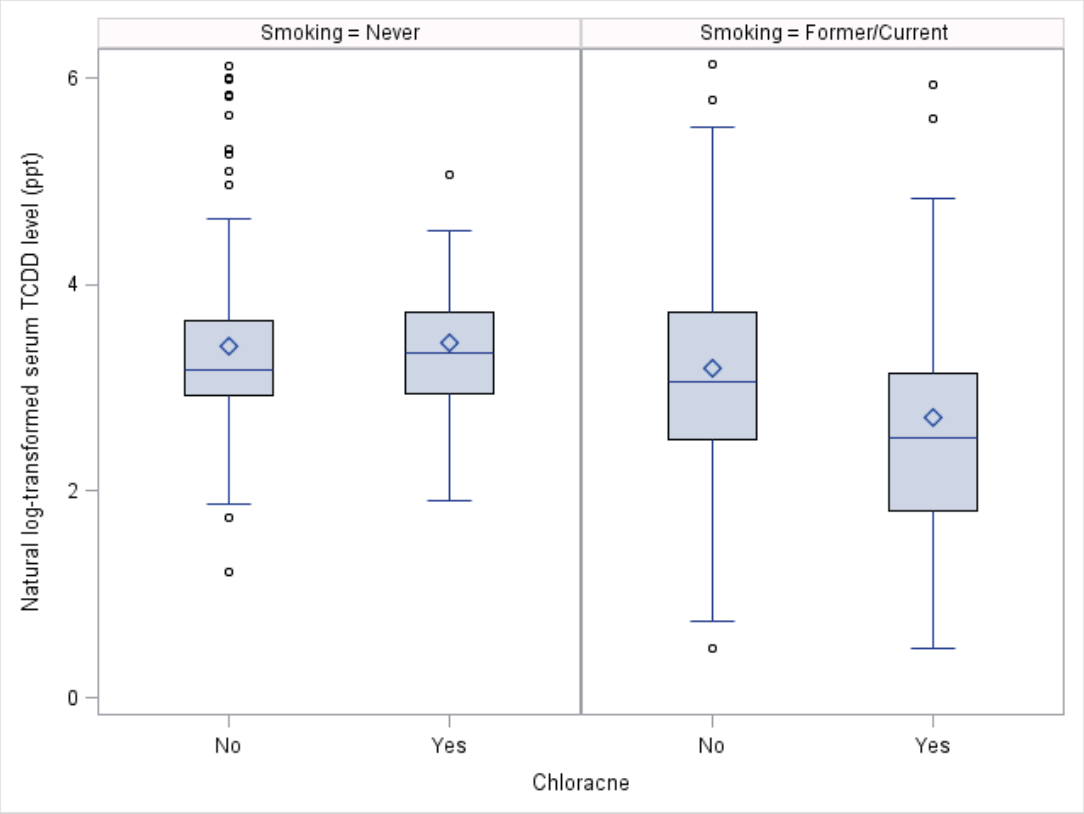

Figure 9. Boxplot of natural log-transformed serum TCDD and chloracne development, stratified by present-day smoking status.

TABLE XVI

GEOMETRIC MEAN TCDD AND TEQ SERUM LEVELS BY SMOKING CATEGORIES

	n	Pack years[c] mean	TCDD[d] mean	(95% CI)	TEQ[d] mean	(95% CI)
All Workers[a]						
Current smokers	43	38	15.5	(10.3–23.2)	50.8	(37.9–68.1)
Former smokers	41	28	28.8	(20.8–39.8)	78.9	(62.6–99.4)
Never smokers	134	0	30.2	(25.8–35.3)	81.4	(71.4–92.9)
Subjects with Chloracne[b]						
Current smokers	19	43	11.7	(6.6–20.7)	41.8	(27.1–64.3)
Former smokers	12	26	22.5	(10.8–46.9)	74.2	(45.3–121.4)
Never smokers	14	0	31.1	(19.7–49.1)	75.9	(55.5–103.8)
Subjects without Chloracne[b]						
Current smokers	23	34	18.6	(10.1–34.5)	58.8	(38.0–90.8)
Former smokers	26	28	31.1	(20.7–46.7)	78.9	(58.4–106.6)
Never smokers	116	0	30.2	(25.4–35.9)	82.8	(71.5–95.8)

[a] Includes all sampled plant workers (n=218).

[b] Chloracne status was unknown in eight plant workers.

[c] Duration of smoking or packs smoked unknown for one current and one former smoker.

[d] Measured in parts per trillion (ppt), lipid-adjusted.

[e] Geometric mean and 95% confidence interval.

In the crude analysis of the association with chloracne status (Table XVII), expression of the AhR and ARNT genes were found to be 1.1-fold higher in those who developed chloracne as compared with those who did not, though these increases were not statistically significant. The AhRR expression was estimated to be 1.6-fold higher in those who developed chloracne (p=.029). Table XVIII displays results from the gene expression analysis using logistic regression modeling with model-based standardization. After adjustment for demographic variables, AhR expression was found to be significantly associated with chloracne development, and the association with AhRR expression was borderline significant (Table XVIII). However, these associations were attenuated after adjustment for current smoking status.

TABLE XVII

CRUDE ASSOCIATION BETWEEN EXPRESSION OF SELECTED GENES IN THE AHR PATHWAY AND CHLORACNE STATUS

Gene	Chloracne	n	mean ΔCt	s.d.	median ΔCt	Fold change	p-value
AHR	No	44	3.2	0.5	3.1	1.1	0.669
	Yes	13	3.1	0.6	2.9		
ARNT	No	43	5.6	0.4	5.5	1.1	0.203
	Yes	13	5.4	0.4	5.6		
AHRR	No	43	10.4	0.8	10.4	1.6	0.029
	Yes	13	9.8	1.3	10.1		

[a] ΔCt = difference in cycles to threshold (number of PCR cycles necessary to reach threshold expression level).

[b] Fold change calculated as $2^{(-\Delta\Delta Ct)}$.

[c] Welch-Satterwaite t-test p-values shown.

TABLE XVIII

LOGISTIC REGRESSION MODELS OF THE RELATIONSHIP BETWEEN CHLORACNE STATUS AND RELATIVE GENE EXPRESSION FOR SELECTED GENES IN THE AHR PATHWAY

Relative gene expression[a]	Model 1[b] RR	Model 1[b] 95% CI	Model 2[c] RR	Model 2[c] 95% CI
AhR	0.81	(0.63–0.98)	0.84	(0.69–1.41)
ARNT	0.98	(0.86–1.44)	0.96	(0.84–1.03)
AhRR	0.99	(0.93–1.00)	0.99	(0.93–1.02)

[a] Includes also subjects sampled for gene analysis (n=60); Relative gene expression measured as an increase ΔCt (decrease in Ct indicates an increase in gene expression).

[b] Adjusted for age at hire, gender, and ethnicity.

[c] Adjusted for age at hire, gender, ethnicity and current smoking status.

[d] Estimated from logistic regression model using model-based marginal standardization technique, as described by Localio et al., 2007.

[e] Estimated using bias-corrected bootstrap method described by Mooney and Duval, 1993.

D. **Discussion**

A causative relationship between exposure to chlorinated aromatic compounds and chloracne development has been generally accepted by the scientific community for decades, supported by numerous experimental and observational studies (Suskind, 1985; ATSDR, 1998; Ju and Zouboulis, 2013). Our major aim was to retrospectively identify demographic, behavioral, occupational, and/or physiological factors that might explain why only some individuals with occupational dioxin exposure develop chloracne. In our cohort of chemical factory workers involved in the production of chlorinated phenols, 45 subjects were diagnosed with past or present chloracne. Twenty-one (47%) of these cases were involved in the production of 2,4,5-T at some point during their tenure at the plant (Table XII). It is known that TCDD is a contaminant of the process used to manufacture 2,4,5-T (NTP, 2011). Another chlorophenoxy herbicide produced at the plant, 2,4-D, has been shown to be contaminated primarily by higher chlorinated PCDDs (ATSDR, 1998), though 2,3,7,8-substituted PCDDs and PCDFs were previously identified in 2,4-D from Russia (Fedorov and Myasoedov, 1990; ATSDR, 1998). Approximately 22% of workers in our cohort who were ever employed in the 2,4-D production departments developed chloracne, though this percentage decreased to about 15% when considering only those who exclusively worked with 2,4-D at the plant (Table XIII). Most of the plant personnel worked in multiple areas of the factory over the course of their employment, and thus, experienced varied dioxin exposures. Though information regarding specific chemical exposures could not be obtained for those who worked in departments other than the chlorophenoxy herbicide-producing areas, large variance in current dioxin serum levels suggests that exposures were heterogeneous.

Current and back-extrapolated blood serum TCDD levels in this cohort were not associated with chloracne development, nor were our estimates of cumulative exposure (Table XIV). We estimated cumulative exposure for each job title and department of the plant using current serum TCDD levels, since historical sampling data from the chemical facility was not available to us. Thus, it is not surprising

that these three exposure measures showed a similar relationship with the disease. Previous study of dioxin-exposed individuals suggests that serum TCDD concentrations are not necessarily correlated with the occurrence or severity of chloracne (IOM, 2009). For instance, in one study of German factory workers occupationally exposed to dioxin during the production of 2,4,5-T and other chlorophenols, researchers observed chloracne cases among those with the lowest adipose tissue dioxin concentrations (Beck et al., 1989). In another study of seven workers (five of whom developed chloracne) involved in the manufacture of 2,4,5-T at the same chemical plant highlighted in this paper, one of the chloracne cases had the lowest TCDD blood concentration (Schecter et al., 1993). Similar findings have been noted among environmentally exposed individuals of the Seveso, Italy, cohort (Mocarelli et al., 1991).

However, presence at the plant during the timeframe 1964–1968, when several industrial accidents in the 2,4,5-T department reportedly occurred, was significantly predictive of chloracne diagnosis (Table XIV). In fact, all of the workers involved exclusively in the manufacture of chemicals other than 2,4,5-T who developed chloracne were present at the plant at some point between 1964 and 1968. This may suggest that the industrial accidents that occurred in the 2,4,5-T production department were not contained to that area, but rather contaminated the entire plant. Nevertheless, several of those who did not develop chloracne were also present at the time of the accidents, which encouraged further exploration of other potential contributing factors.

A wide variety of demographic, behavioral, and physiological information collected from surveys and physical examinations were found to be unrelated to the diagnosis of chloracne. However, we observed a striking difference in self-reported smoking status between those factory workers who were diagnosed with chloracne and those who were not. The risk of chloracne development was elevated in both former smokers (RR=2.80, 95%CI: 1.04–6.95) and current smokers (RR=4.16, 95%CI: 2.08–10.15), after adjustment for age at hire, gender, ethnicity, and whether present during the 1964–1968 time period (Table XV). Among those who worked exclusively in the 2,4,5-T production department, four of

the six (67%) who were diagnosed with chloracne were self-reported former or current smokers versus just one of ten (10%) who were not diagnosed with the skin condition. Such dramatic differences in smoking habit between dioxin-exposed individuals with and without chloracne were briefly reported in one other study of laborers involved in the production of 2,4,5-T at a chemical plant. The author of this study observed that 16.5% of chloracne cases in the cohort never smoked, versus 39.3% who never smoked in the non-chloracne group (Suskind, 1985). Use of protective gear, washing, and bathing was further found to be more frequent in the non-chloracne group described by Suskind (1985), though we did not investigate these specific behaviors in our cohort.

In general, current serum TCDD concentrations were lower in those who developed chloracne compared to those who did not, though this difference was not statistically significant after adjustment for demographic and behavioral factors (Table XIV). It is possible that the reason for the lack of correlation between blood TCDD levels and chloracne occurrence may have less to do with the size of the acute exposure and more to do with the timing of blood sampling. The hamartomas that manifest in chloracne skin lesions accumulate TCDD and express cytochrome P450 at high levels, which is thought to reduce systemic toxicity through increased metabolism (Tan and Wahli, 2014).

It is also possible that cigarette smoking contributed to the relatively lower serum TCDD levels in chloracne cases. Other studies have demonstrated an inverse relationship between smoking and serum TCDD (Chen et al., 2005; Garabrant et al., 2009). As shown in Table XVI, current smokers in our cohort had much lower serum TCDD concentrations than former smokers and never smokers. Figure 9 further suggests that the lower TCDD serum levels observed in chloracne patients is only apparent in current and former smokers. It has been hypothesized that the PAHs found in cigarette smoke may further induce cytochrome P450, which is involved in the metabolism of dioxins (DeVito et al., 1995; Chen et al., 2005). Additionally, Flesch-Janys et al. (1996) estimated faster PCDD and PCDF decay rates in smokers, as compared to former and never smokers.

Furthermore, the link between smoking and increased risk of chloracne development may be biologically plausible. Several studies have implicated the AhR pathway in chloracne pathogenesis (Tauchi et al., 2005; Tang et al., 2008; Ju et al., 2011). The TCDD-mediated production of reactive oxygen species have further been shown to accelerate keratinocyte differentiation (Kennedy et al, 2013). More recently, a group of researchers found that constitutively active Nrf2 in keratinocytes of mice led to the development of large keratinized skin cysts combined with the loss of sebaceous glands, a pathology reminiscent of that seen in patients with chloracne. According to the same study, treatment of human keratinocytes with TCDD resulted in upregulation of Nrf2 and its targets via activation of the AhR (Schäfer et al., 2014). The Nrf2 is a transcription factor that coordinates the cellular response to oxidative stress. It is therefore unsurprising that cigarette smoke can induce its activity, since this exposure inundates cells with free radicals, reactive oxygen species, reactive nitrogen species, and other xenobiotic compounds (Müller and Hengstermann, 2012). There are also cigarette smoke constituents that can activate the AhR, including PAHs and BaP (Kitamura and Kasai, 2007).

Thus, there seem to be at least two distinct mechanisms through which induction of the Nrf2 pathway can occur, one related to the AhR and one related to direct oxidative stress. The TCDD exposure can stimulate the AhR, while constituents in cigarette smoke can induce the Nrf2 through either path. Molecular studies of the interplay between TCDD and cigarette smoke exposure on these metabolic pathways may provide crucial insight into the mechanisms through which chloracne risk differs between individuals.

In our study, we assessed the relative expression of several genes in the AhR pathway between those with a history of chloracne and those without. Years after occupational exposure had ceased, we observed very few significant differences in gene expression. Expression of the AhRR was significantly higher in those diagnosed with chloracne, though this difference diminished after adjustment for current smoking status (Table XVII). Our findings suggest that, for this particular acute condition, it is

possible that gene expression differences cannot be observed using blood samples collected years after exposure.

It is worth noting that the prevalence of hypertension, as determined by physical examination at the time of study recruitment, was associated with a chloracne diagnosis. Twenty-five of the 36 men who were diagnosed with chloracne had hypertension in 2009 (69%), versus 51% in men who did not develop chloracne (chi-square p=.07). All 9 women who were diagnosed with chloracne had hypertension in 2009 (100%), compared to 68% of women without chloracne (Fisher's Exact Test p=.04). Similar findings were reported in a study of Taiwanese individuals poisoned by the consumption of PCB-contaminated rice-bran oil in the late 1970s. A significant association between chloracne development and hypertension was reported for women but not for men in this "Yucheng" cohort. Though this relationship was not addressed in our study hypothesis, these results warrant further investigation.

1. **Limitations**

The chief limitation of this analysis was the timing of subject enrollment. Workers were recruited in 2009—between 18 and 19 years after the last year of work at the plant, on average, and 41–45 years after the industrial accidents occurred in the 2,4,5-T production department. Chloracne results from acute exposure to dioxin, with symptoms occurring within weeks of exposure (ATSDR, 1998). The half-life of TCDD in humans varies from cohort to cohort and from individual to individual. Factors such as BMI, age, gender, and initial concentration can affect the rate of excretion (IOM, 2009). As discussed previously, smoking may also affect the metabolism of TCDD (Chen et al., 2005; Garabrant et al., 2009). Inter-individual differences in metabolism and potentially lengthy periods of time since chloracne development may have muddied our estimates of association with serum TCDD concentration. We were unable to make solid conclusions with regards to serum TCDD levels at the time of chloracne

development. Similarly, we found very few significant differences in gene expression between the chloracne and non-chloracne groups, likely as a result of the time lapse since acute exposure.

Additionally, the time at which chloracne first developed could not be ascertained with accuracy during the dermatologist examination. Likewise, very few subjects with chloracne reported the year during which their disease manifested. However, based on previous study of workers at this plant and communication with employees, we ascertained that a large number of chloracne cases occurred in the 1960s, which supports our finding that being present at the factory from 1964 to 1968 was a significant risk factor for disease development.

The measurement of smoking habits was also imperfect, given that this information was collected years after exposure. Though duration and amount of smoking was reported by most participants, the years during which they smoked were rarely available. Thus, we cannot say for certain whether those who developed chloracne were more likely to have smoked concurrently with TCDD exposure. However, we found that current smokers smoked for a greater number of pack-years than former smokers, and the association with chloracne was stronger in current smokers. Current smokers were more likely to have smoked during years in which they were exposed to TCDD.

Lastly, we were unable to directly assess differences in Nrf2-activated genes between those who were diagnosed with chloracne and those who were not, since we did not select these genes for study during the design phase of the project. However, blood samples from subjects included in this occupational cohort are archived. Future studies of this cohort can thus investigate the differential expression of genes regulated by the Nrf2, particularly when it comes to chloracne. Additionally, single nucleotide polymorphisms and other mutations have been estimated to occur at a rate of approximately 1 per 72 base pairs (Cho, 2013). In the future, we hope to investigate whether Nrf2 genotype can help to explain the observed individual variation in susceptibility to chloracne in dioxin-exposed cohorts.

2. Conclusions

Cigarette smoking was identified as a potential risk factor for chloracne development in this occupationally exposed cohort of Eastern European chemical plant workers. Given the drug metabolism pathways activated by cigarette smoke and TCDD, this relationship seems biologically plausible. Chloracne diagnosis was also significantly associated with whether subjects were present at the plant during likely high exposure years (1964–1968), a measure of acute TCDD exposure. Future experimental investigations of the interplay between TCDD and cigarette smoke exposures would be prudent.

This study also highlights important methodological considerations in chloracne investigation. Our results suggest that blood samples collected years after chloracne development may not be ideal for assessing exposure-response relationships. However, because occupational dioxin exposures have declined over the last several decades and large-scale environmental contamination is relatively rare, retrospective analyses will continue to be crucial to understanding the effects of dioxins in human populations. Our findings bring attention to the continued importance of traditional exposure reconstruction techniques, despite the increased focus on biomarkers in epidemiology.

VI. CONCLUSIONS

A. Study Implications

The research questions selected for study, the results of which have now been described, sought to fill gaps in our collective knowledge regarding the mechanisms through which dioxins exert their toxicity in humans. These questions were addressed by retrospectively investigating relationships between blood dioxin levels and several physiological/biological outcomes in two cohorts. Analytical methods were selected so as to maximize the validity of the study results.

The cohorts examined in these studies are unique for several reasons. The occupational cohort of chemical workers provides a rare opportunity to investigate the effects of high-level exposure to a complex mixture of dioxins. Blood serum dioxin levels in these subjects were still elevated, on average, years after exposure had ceased. The relatively high proportion of women included in this cohort of factory workers (43%) was also atypical, allowing for more robust control for and assessment of possible gender effects. The population-based control cohort of individuals from a different city was also distinctive, in that these subjects harbored a wide range of blood serum NDL-PCB levels. The diverse chemical body burdens allowed for the assessment of chemical interactions not previously studied in human observational studies.

The study of the relationship between PCB 126 (a DL-PCB) and t(14;18) prevalence and frequency demonstrated a significant association among former and current smokers. Given the established role of the chromosomal translocation as an early step in the progression toward malignancy, this positive association suggests a mechanism through which dioxins may induce susceptibility to follicular lymphoma, supporting previously observed associations between dioxin exposure and NHL in other observational cohort studies. This finding has both risk assessment and regulatory implications. Though scientific bodies have concluded that there is a plausible link between dioxin exposure and NHL (IOM, 2009), there is still considerable debate. The results presented in this

document provide compelling evidence of a plausible link with one subtype of NHL—follicular lymphoma.

Serum dioxin levels were found to be positively associated with the relative gene expression of the AhR itself, though not with the expression of genes regulated by the AhR, as presented in the second study described in this document. Furthermore, concurrent serum levels of specific congeners of NDL-PCBs were found to modify the relationship between dioxin levels and AhR expression. The modern human body is a highly efficient machine, crafted over the course of hundreds of thousands of years. Small adaptive changes and random mutation allowed us to evolve with our environment through a slow trial and error process—those changes that affected our phenotype in ways that improved our probability of survival and reproduction were passed on to the next generations. Given the vast amount of time over which these processes took place, it is not surprising that our physiology is highly complex. The AhR, a gift from species that existed long before humans, has allowed us to effectively respond to environmental change over time (McIntosh et al., 2010). The AhR serves a critical function in xenobiotic metabolism and is thus an important target for research in the field of environmental health. A better understanding of the mechanisms by which it is regulated and by which it regulates other functions will greatly enhance the risk assessment process. To our knowledge, the modifying effects of NDL-PCBs on dioxin-induced AhR expression have not been previously demonstrated in observational human studies. Such investigation is critical, as the chemical exposure experience of humans in the real world is complex. The findings presented in this document also highlight areas for future study.

The results presented for the study of chloracne diagnosis in the occupationally exposed cohort reveal a potentially important relationship between dioxin exposure and a behavioral factor—cigarette smoking—in the development of this skin condition. The findings support recent reports regarding the role of the Nrf2, a transcription factor that facilitates the body's response to oxidative stress, in the development of chloracne-like skin hamartomas (Schäfer et al., 2014), as smoking is an agonist in this

response pathway. Our study further suggests a novel framework for thinking about chloracne not just as a toxic response, but also as a mechanism for detoxification, as evidenced by the lower blood dioxin levels in our chloracne-diagnosed subjects. The findings highlight exciting prospects for future experimental study of these pathways.

Findings presented in this document additionally have direct and immediate implications for public health, particularly with regards to the modifiable behavior of cigarette smoking. In our studies of both the occupationally exposed and environmentally exposed cohorts, smoking was found to modify the effects of dioxins (including DL-PCBs) on more than one health endpoint. Though the decreasing smoking rates in the United States over the last few decades represent a public health triumph, more must be done. This is especially pressing, given that we are now endemically exposed to various chemicals in the environment as a result of industrialization. Moreover, smoking rates are still high in developing countries. The studies described above represent a rallying call for those in the public health, medical, and political spheres to continue to devise creative smoking cessation strategies and methods to limit the emission of hazardous chemicals into the environment.

B. **General Limitations**

There are several potential limitations to the study design and data collection that must be considered in this set of analyses. First, we were only able to obtain blood serum data from 218 of the 368 factory workers sampled. This introduces a potential selection bias, present when the probability of inclusion in the selected sample among individuals differs based on characteristics relevant to the study (Rothman et al., 2008). During the data collection phase, an effort was made to recruit the most highly exposed workers first (i.e., those who were involved in the manufacture of TCPand its esters). Those recruited later were more likely to have been involved in the manufacture of 2,4-D, and thus likely experienced lower levels of TCDD exposure (ATSDR, 1998). The UIC was only able to obtain data from

blood samples of the first consecutive 218 workers recruited. However, measurement of current blood dioxin levels for all subjects allows for substantiation of the comparability of groups, and thus should have maintained the validity of the comparisons of interest. The potential for selection bias also arises from missing data (e.g., unanswered questions on a survey), though it seems that the data relevant to the studies described above were very close to complete. One other source of selection bias may have been related to choice of comparison group. As shown previously, DL-PCB and NDL-PCB levels in both the exposed and unexposed groups were very similar. This could have skewed results of investigations into the toxicity of dioxins. However, this may also be a strength of the study, in that the levels of PCBs and dioxins in the study participants vary widely. For instance, this allowed us detect possible interactions between dioxins and PCBs.

Information bias results when, after subjects to be compared have been identified, information about them to be used for analysis is erroneous. This can result in misclassification of study subjects (Rothman et al., 2008). The second set of possible limitations in the studies described above relates to this type of bias. For one, self-reported medical and occupational history information collected was based on recall, though actual assignment of workers to job classes and titles was based on written work history records—the system for gathering such information was quite detailed and accurate. The major source of potential information bias in these studies likely concerned the exposure assessment. In particular, for the exposed group of workers, blood samples were obtained years after primary exposure periods. Though dioxins and PCBs are highly persistent in the body, there were likely individual-level differences in elimination rates. Differential half-lives among dioxin congeners further complicate this issue. The similarity between the exposed and unexposed groups in DL-PCB body burden may have also resulted in exposure misclassification. Nevertheless, our preliminary analysis demonstrated that workers in the highest exposure classes did indeed have the highest blood dioxin levels. Another potential for bias came with the assumption of additive toxicity of dioxin congeners when considering serum TEQ. Yet

this issue was assessed in the second study, the findings of which suggested that concurrent exposure to other chemicals may modify the effects of dioxin in the human body. Misclassification of subjects by study outcome was also a danger, particularly in the third study. In that investigation, past chloracne development was assessed by a dermatologist. Though the inclusion of a trained professional in ascertaining past chloracne status likely provided stronger evidence than patient recall alone, individual differences in severity may make the condition difficult to identify (both for the patient and the dermatologist).

As with all observational epidemiological studies, confounding was also an important source of bias to consider. Though the identification of true causal relationships is theoretically impossible in both experimental and observational study (as per the counterfactual theory of causation), the goal of many epidemiological studies is to estimate associations that approximate these causal relationships. When one attempts to assign causation in such a study, one implicitly makes the assumption that all confounding has been accounted for (Rothman et al., 2008). The study design in this research made an attempt to account for as many contributing variables as possible.

Another major issue to address was the statistical power of the studies, particularly when it came to the gene expression analyses. Determination of sample size for such studies depends on several factors, including number of genes (84 in this case), the probability of random noise in expression, the FDR, and the proportion of genes that are likely to be significantly different. The power analysis used to determine how many subjects to randomly sample for RT-PCR tests used an FDR of 10%, which is very conservative. We expected to detect differentially expressed genes, when at least 15% of the genes in the assay were in fact differentially expressed, with at least 80% power.

Finally, the choice of cells tested may have affected our ability to draw valid and generalizable conclusions. For instance, it is possible that certain genes of interest may not be expressed to any great extent in human lymphocytes (e.g., certain P450 enzyme genes may not be as strongly expressed in

lymphocytes as they are in liver cells). One may also question whether differential gene expression in the lymphocytes among groups with different exposure profiles would be similar in other cell types that express those genes. However, because of the relative ease with which blood can be obtained from patients, it is important to study whether biomarkers of dioxin exposure can be detected in these cells.

Despite the limitations of this research project, these data will provide a unique perspective on dioxin toxicity that will drive further inquiry. The studies presented here provide new insight into the mechanisms through which dioxins exert their toxicity and represent a small step forward in the journey toward understanding how humans interact with the environment in which they live.

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VITA

NAME:	Peter Seth Ruestow
EDUCATION:	BA, Economics, University of Michigan, Ann Arbor, Michigan, 2004
	MPH, Health Management and Policy, University of Michigan, Ann Arbor, Michigan, 2006
	PhD, Environmental and Occupational Health Sciences, University of Illinois at Chicago, Chicago, Illinois, 2015
TEACHING:	Department of Epidemiology and Biostatistics, University of Illinois at Chicago, Epidemiologic Computing, Teaching Assistant, 2012–2013
	Department of Epidemiology and Biostatistics, University of Illinois at Chicago, Advanced Quantitative Methods in Epidemiology, Teaching Assistant, 2013
	Department of Environmental and Occupational Health Sciences, University of Illinois at Chicago, Lecture: Applications of the Illinois Occupational Surveillance Program (IOSP): Designing epidemiological studies using existing State databases, 2013
	Department of Environmental and Occupational Health Sciences, University of Illinois at Chicago, Lecture: Microsoft Excel (not for dummies): Skills to maximize your graduate school achievement (and your marketability too), 2013
HONORS:	National Institute of Occupational Safety and Health Trainee, 2012–2014
PROFESSIONAL MEMBERSHIP:	American Public Health Association
PRESENTATIONS:	Ruestow, P., Duke, T., Finley, B., & Pierce, J. Evaluation of the impact of the "Restraining Line Rule" on the incidence of total and head injuries occurring during NFL kickoffs. American Industrial Hygiene Conference & Expo (AIHce), 2015. Salt Lake City, UT.
	Ruestow, P., & Forst, L. Racial disparities in discharge location following hospitalization for traumatic falls among the elderly. APHA Annual Meeting and Expo, 2012. San Francisco, CA.

VITA (CONTINUED)

PRESENTATIONS: Dookeran, K. A., Sciupokiene, E., Mackeviciute-Naujokas, A., Elfarra, G., Rogowski, W. A., Patel, J., Ruestow, P., & Zaren, H. A. Minority underserved women treated for breast cancer are at increased risk for poor mental health outcomes. AACR Proceedings, 2003. MBCCOP, Cook County Hospital, Chicago, IL.

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