Environmental Exposures and Sex Chromosome Abnormalities in Human Sperm

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Dedication

To my everlasting Father who loves me unconditionally.

To all the parents who have lost a child due to unexplained chromosomal abnormalities.

To Mr. Rafael Correa Celis (1924-), an amazing husband, father of five children. Because your vision for our family has always been anchored in the value of education, your legacy and love is alive in each of our accomplishments. May I never forget your teachings about the most valuable gifts in life.

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Abstract of Dissertation

Environmental Exposures and Sex Chromosome Abnormalities in Human Sperm

Despite evidence that US infertility is increasing and environmental exposures may be impacting human reproduction, the reproductive effects of environmental chemicals remain understudied, particularly in men. Some genetic defects and disorders are transmitted by spermatozoa and several chromosomal abnormalities come from the father. In germ cells, failure of sex chromosomes (X or Y) to separate properly during meiosis (I or II) results in an extra or missing chromosome, known as aneuploidy and these errors in chromosome number are the most common chromosomal abnormality in humans. Disomy, or an extra X or Y chromosome, is the most frequent form of aneuploidy observed in human sperm. Limited information has been published about exposures to endocrine-disrupting chemicals (EDCs), such as organophosphate (OP) and pyrethroid (PYR) pesticides, and their association with altered frequency of sperm chromosomal abnormalities. Although extensive literature on pesticide toxicology is available, many hormone-mediated effects, specifically on the testes, are not well understood. This dissertation involved three separate investigations.

Study subjects were men from a parent study assessing the impact of environmental exposures on male reproductive health at the Massachusetts General Hospital (MGH) Fertility Center (2000-2003). Multi-probe fluorescence in situ hybridization (FISH) for chromosomes X, Y, and 18 was used to determine XX18, YY18, XY18 and total sex chromosome disomy in sperm nuclei. Urine was analyzed for common PYR and OP metabolites. Poisson regression models were used to calculate incidence rate ratios (IRRs) for each disomy type by the exposure of interest, controlling for age, race, BMI,

smoking, specific gravity, total sperm concentration, motility, and morphology. Sensitivity analyses were also conducted. Interactions between PYR and OP metabolites and associations with each disomy outcome were examined in the adjusted models.

First, we investigated environmental exposure to PYRs and their association with sperm disomy among adult men (n=181). Inverse associations were observed between PYR exposure levels and most disomy outcomes. Second, a similar investigation was conducted with OP pesticides (n=159). Increased and inverse IRRs were associated with specific OP metabolites. Total sum of OP metabolites concealed individual associations for specific OP metabolites. Dose-response relationships between OP exposure levels and disomy appeared nonmonotonic, with an increase in disomy rates between the second and third exposure quartiles and without additional increases between the third and fourth exposure quartiles. Finally, we examined the hypothesis that pesticide mixtures (OP/PYR) and their potential interactions alter associations with sperm disomy (n=159). Significant interactions were identified between OP/PYR mixtures and sperm disomy. Increased IRRs, higher than the rates previously detected for each individual chemical class, were observed when evaluating the associations between disomy outcomes and pesticide mixtures. Nonmonotonic relationships between pesticide mixtures and disomy demonstrated that further modeling work is needed to: a) determine the optimal method to evaluate pesticide mixtures with different modes of action; b) investigate interactions using non-logistic models; and c) identify the mechanisms by which EDCs affect health reproductive outcomes. Our findings showed that PYR and OP pesticides altered the associations of sperm disomy, suggesting that the impacts of EDCs on testis function need further characterization in epidemiologic studies.

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Chapter 1: Introduction

Despite evidence that infertility is increasing in the US and environmental exposures may be impacting the reproductive health of offspring (ARHP, 2010), the reproductive impacts of commonplace chemicals remain understudied, particularly in men (Swan et al., 2000; Travison et al., 2007; Woodruff et al., 2008). The World Health Organization (WHO) reports that at least 80 million people worldwide were estimated to be affected by infertility; among all infertility cases, 10% cannot be explained medically (WHO, 2002).

In the Unites States, each year about 2 million couples who want to have children are infertile (CDC, 2013); infertility affects about 15-17% of couples trying to become pregnant (Thoma et al., 2013). Infertility is defined in the CDC's National Survey of Family Growth 2006-2010 as being unable to conceive after at least 12 consecutive months of unprotected sex. In addition, about 6.7 million conceptions are lost before gestation is complete. The American Society of Reproductive Medicine estimates that, in approximately 40 percent of infertile couples, the male partner is either the sole cause or a contributing cause of infertility (ASRM, 2014). Furthermore, there is growing evidence that a number of genetic defects and disorders are transmitted by spermatozoa. Several chromosomal abnormalities, including the number of sex chromosomes, come from the father (Hassold and Hunt, 2001). Aneuploidy, (i.e., an extra or missing chromosome), is the most common chromosomal abnormality in humans and results from the failure of chromosome pairs to separate properly during cell division (i.e., non-disjunction). In germ cells, errors in chromosome segregation during meiosis (I or II) result in structural aberrations and imbalances in chromosome number. Disomy is the most frequent

aneuploidy observed in human sperm (Figure 1.1), yet the exact causes of non-disjunction are unknown.

Aneuploidy

Although most aneuploid conceptuses perish in utero, approximately half of all spontaneous abortions are associated with pre-existing chromosomal abnormalities (Jacobs, 1992; Lebedev et al., 2004). Sex chromosomes are particularly susceptible to aneuploidy; most aneuploidies found at birth involve an abnormal number of X or Y chromosomes (Martin et al., 1991). Sex chromosome aneuploidy has been identified in at least 5% of all clinically recognized pregnancies (Hassold and Hunt, 2001).

Studies have examined the frequency and distribution of chromosomal abnormalities in spermatozoa to establish background levels of sperm aneuploidy in normal men (Templado et al., 2005, 2011). The study of the paternal role in sex chromosome aneuploidy is crucial because paternal-originated chromosomal errors account for a large percentage of reproductive health problems (e.g., Klinefelter Syndrome (47, XXY), Turner Syndrome (45,X)) (Olson and Magenis, 1988; Chandley et al., 1991; Crow et al., 2001). Children with sex chromosomal abnormalities, characterized by Kleinfelter's and Turner's syndromes, may have reproductive disorders, behavioral difficulties and/or reduced intellectual capabilities compared to their siblings or other children with normal chromosome complements (Boyd et al., 2011; Martin, 2006). There is evidence from European birth defect registries showing that the prevalence of chromosomal abnormalities in infants measured during the first 28 days after birth increased between 1967-1988 (Morris et al., 2008). The authors suggest that there may be underlying

environmental causes affecting spermatogenesis (Morris et al., 2008). Similar birth registry data are not available for the US.

Scientific reviews have aimed to characterize environmental chemical exposures and their impact on men's reproductive health (Foster, 2008; Mruk and Cheng, 2011; Diamanti-Kandarakis et al., 2009; Woodruff, 2011; Zoeller et al., 2012; WHO, 2013). Although it is well demonstrated that toxicants adversely affect germ cell DNA integrity (Mruk and Cheng, 2011), the exact causes of aneuploidy and the specific windows in which adverse exposures impact the human spermatogenic cycle are not well understood (Herrera et al., 2008; Axelsson et al., 2010; Ashton Acton, 2013).

Biological Mechanism

Significant advances have been made in the field of male reproductive biology which unlock at least in part the molecular and biochemical events that regulate spermatogenesis in the mammalian testis (Cheng et al., 2008; Cheng and Mruk, 2010). Spermatogenesis is a highly complex and regulated process by which spermatozoa are produced from male primordial germ cells through mitosis and meiosis (I or II). It is highly dependent upon optimal conditions for the process to occur correctly, and is essential for sexual reproduction (Figure 1.2). Errors during the first meiotic division (meiosis I) result in XY disomy, whereas errors in the second meiotic division (meiosis II) result in XX or YY disomy (Griffin et al., 1995; Figure 1.2). Sperm maturation models have suggested sperm damage is a consequence of: a) sensitivity in various phases of spermatogenesis, b) exposure to environmental chemicals, and/or c) random error during sperm production (Figure 1.3). There is a likelihood that paternal exposure

to chemicals may alter the epigenetic reprogramming during spermatogenesis and the early stages of sperm development (Marchetti and Wyrobeck, 2005).

The Role of Reproductive Hormones

Reproductive hormones are critical to the initiation and continuation of spermatogenesis and for male fertility (Dohle et al., 2003). Recently studies have explored the effects of pollutant exposure on the processes of spermatogenesis where chemicals can interfere with cell signaling via direct/indirect "hormonal" and/or oxidative stress related pathways disrupting the regulation of testicular development and functions (Yeung et al., 2011). There is emerging evidence that environmental chemicals can adversely affect men's health (Meeker et al., 2010), including spermatogenesis and the occurrence of chromosomal aberrations through mechanisms of endocrine hormone modulation (Martin, 2006; WHO, 2013). If cells in the male testis are exposed to low doses of estrogenic chemicals, an effect on estrogen receptor expression may provide a molecular mechanism for spermatogenesis disruption (Taylor et al., 2010).

Endocrine Disrupting Chemicals

Environmental chemicals are known to disrupt hormone signaling that may interfere with recombination sequences (Eil and Nisula, 1990). Endocrine disrupting chemicals (EDC) are compounds that alter the normal functioning of the endocrine system of both wildlife and humans. EDCs include a wide range of chemical classes which display more than one mechanism of action, structure and potency (US EPA, 2013; NAS, 2014). There are a number of mechanisms through which endocrine disruptors can modulate endocrine systems and potentially cause adverse effects in humans (Mnif et al., 2011; Balabanic et

al., 2011; Sharpe, 2009). Hormone signaling disruptions may be risk factors for aneuploidy. Follicle-stimulating hormone (FSH) has been shown to be associated with increases in the frequency of sperm aneuploidy among men with low sperm concentration undergoing intracytoplasmic sperm injection (ICSI) (Faure et al., 2007). Based on the reported decreases in sperm concentration, sperm motility and morphology in men seeking infertility treatment, it has been suggested that environmental chemicals with estrogenic properties, such as pesticides, are negatively affecting male fertility (Perry et al., 2008; Phillips and Tanphaichitr, 2008; Phillips and Foster, 2008; Diamanti-Kandarakis et al., 2009; Woodruff, 2011).

Pesticide Exposure

Pesticides are just one class of environmental chemicals that have been identified as potential endocrine disruptors. These chemicals are used extensively in both agricultural and residential settings. They have been associated in humans with adverse effects in hormone functions and levels (Meeker et al., 2006, 2009; Lacasaña et al., 2010), semen quality (Lifeng et al., 2006; Xia et al., 2008; Ji et al., 2011; Toshima et al., 2012); decreased semen volume and sperm count (Yucra et al., 2008; Recio-Vega et al., 2008), lower sperm concentration (Perry et al., 2007b), abnormal morphology and decreased sperm motility (Hossain et al., 2010), DNA damage and fragmentation (Meeker et al., 2004b; Xia et al., 2004; Meeker et al., 2008, Ji et al., 2011), sperm chromatin structure alteration (Sanchez-Pena et al., 2004), and sex chromosome disomy in human sperm (Young et al., 2013; Radwan et al., 2015). Thus, there is the need to further understand the role of environmental exposures to pesticides and their relationship with sex

chromosomal abnormalities, especially given the widespread exposures to these contaminants in the general population.

Research Aims & Research

This research aims to:

1) identify environmental risk factors contributing to altered frequency of sperm sex chromosome abnormalities among adult men;

2) evaluate the relationship between contemporary pesticide exposures and sperm aneuploidy (XX18, YY18, XY18 and total sex chromosome disomy); and,

3) explore the health effects of pesticide mixtures and document potential interactions.

This dissertation research project involved three separate investigations. First, we investigated environmental exposure to pyrethroid (PYR) pesticides and their association with altered frequency of sperm sex chromosome disomy among a large sample of adult men. Second, we examined environmental exposure to organophosphate (OP) pesticides and their association with the frequency of human sperm chromosomal abnormalities. Limited information has been published to date about the secondary mechanisms and potential effects of organophosphate pesticides and their association with sperm abnormalities. Finally, we investigated environmental exposure to pesticide mixtures (PYR and OP) and their association with altered frequency of sperm sex chromosome disomy among adult men. The specific goal of the third study was to explore the implications of pesticide mixtures and/or associations that each chemical would not produce individually, and which may account for some unexplained human health effects.

The next three chapters present results from each of these studies. Each chapter provides the background of the issue, the methodology used, the study findings, conclusions and recommendations for future work. The final chapter of this dissertation presents the overall conclusions from this investigation and provides recommendations for the direction of future research.

Figure 1.1. Sperm Sex Chromosome Disomy.

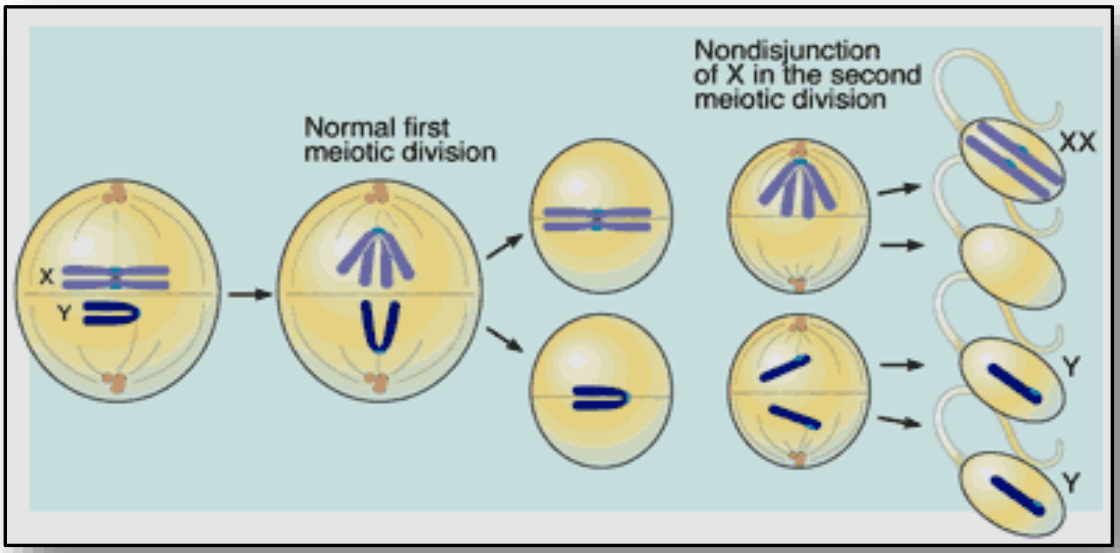


Figure 1.2. Spermatogenesis Biological Mechanism.

(Queen Mary University of London Human Reproduction Course Materials)

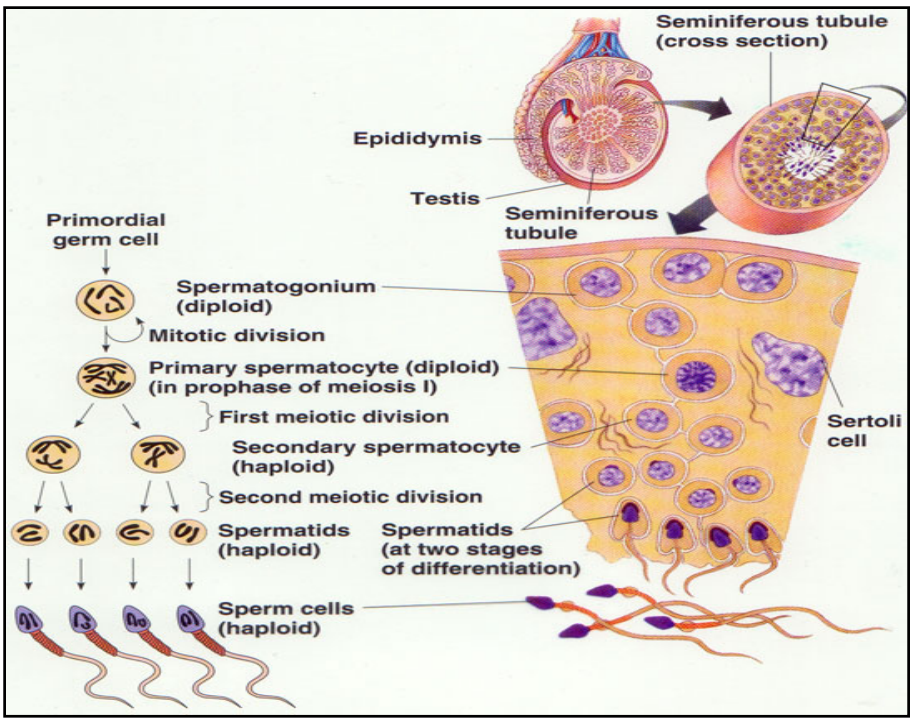
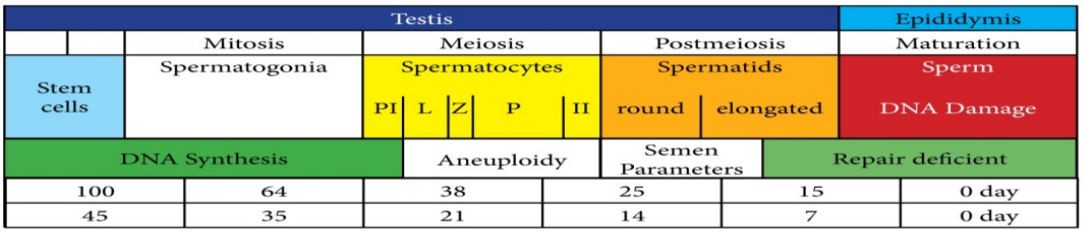


Figure 1.3. Biological Model of Sperm Maturation.

(Adapted from Marchetti and Wyrobeck, 2005)



*PI: preleptotene; L: leptotene; Z: zygotene; P: pachytene; II: metaphase II.

Chapter 2: Environmental Pyrethroid Exposures and Sperm Chromosomal Abnormalities

Introduction

Reproductive problems are increasing among the US population, specifically human infertility due to male factors (Swan et al., 2000; Travison et al., 2007; Woodruff et al., 2008). Previous researchers have attempted to characterize environmental exposures and their impact on men's reproductive health. However, the role of environmental contaminants, particularly endocrine disrupting chemicals (EDCs) such as pyrethroid (PYR) pesticides, and their relationship with chromosomal abnormalities is still not well understood.

Even though chromosomal abnormalities (e.g., aneuploidy) are rare, they are responsible for a portion of reproductive problems, such as congenital abnormality or pregnancy loss. Aneuploidy (i.e., an extra or missing chromosome) results from the failure of chromosome pairs to separate properly. Sex chromosomes are particularly susceptible to aneuploidy; most aneuploidies at birth involve an abnormal number of X or Y chromosomes (Martin et al., 1991). Researchers have studied the frequency and distribution of numerical and structural chromosomal abnormalities in spermatozoa in order to establish baseline values of the sperm chromosome abnormalities in normal men (Templado et al., 2005, 2011). Specifically, disomy is the most frequent aneuploidy observed in human sperm. Disomy results from errors where chromosome pairs fail to separate properly (non-disjunction) during cell division (i.e., meiotic division of spermatocytes) (Figure 1.1).

With the declining use of organophosphate pesticides during the past decade, the use of pyrethroid pesticides has increased in the US. PYR pesticides are a class of synthetic insecticides (i.e., group of man-made pesticides) which are structurally based on the natural pesticide pyrethrum, which can be extracted from Chrysanthemum cinerariaefolium flowers (Soderlund et. al., 2002). Synthetic pyrethroids are adapted from the chemical structures of the extract pyrethrins and act in a similar manner to natural pyrethrum. PYRs are widely used as pest control substances in residential settings as well as in public health programs (e.g., mosquito control), agriculture, and in medical and veterinary products. PYRs are manufactured in over 3,500 registered pesticide products. The National Home and Garden Pesticide Use Survey found that more than 80% of households in the US use pesticides. PYRs are the most frequently used and sold home and garden insecticides in the US (Williams et al., 2008; Barr et al., 2010; Horton et al., 2011). In 2011, the US Environmental Protection Agency (EPA) determined that this growing class of pesticides, including both the naturally occurring pyrethrins and the synthetic pyrethroids, has a common mechanism of toxicity in insects based on their shared ability to interact with sodium channels, leading to changes in neuron firing, and ultimately neurotoxicity (Scollon et al., 2011).

Pyrethroids and their metabolites have been identified in multiple environmental media, for example in soil, aquatic organisms (Erstfeld, 1999), sediment (Gan et al., 2005), food residues (Markovic et al., 2010), and breast milk (Sereda et al., 2009). Urinary levels of PYRs, such as 3-phenoxybenzoic acid (3PBA) and cis/trans-2,2-(dichloro)-2-dimethylvinyl cyclopropane carboxylic acid (DCCA) metabolites, have been measured in the general population (CDC, 2009).

Several studies have evaluated pyrethroid pesticides and their impact on semen parameters and DNA integrity (e.g., Bian et al., 2004; Lifeng et al., 2006; Perry et al., 2007b; Xia et al., 2004, 2008; Meeker et al., 2008; Ji et al., 2011; Toshima et al., 2012). Limited data are available about the specific relationship between PYRs and sperm aneuploidy. Recent studies have evaluated PYR pesticides and their association with human sperm disomy outcomes; however, their findings were not consistent (Young et al., 2013; Radwan et al., 2015).

With the goal of identifying environmental risk factors related to chromosomal abnormalities, this study investigated environmental exposure to pyrethroid insecticides and their association with altered frequency of sperm sex chromosome disomy among a large sample of adult men.

Materials and Methods

Study Subjects

Men (n=181) from a larger study assessing the impact of environmental exposures on semen quality participated in this investigation. In the parent study (n=341), eligible participants were men aged 20-54 from couples seeking infertility evaluation at Massachusetts General Hospital (MGH) Fertility Center between January 2000 and May 2003. Full details of the parent study assessing the impact of environmental exposures on semen have been described elsewhere (Hauser at al., 2003). Approximately 65% of eligible men agreed to participate in the parent study and those declining participation primarily cited lack of time during their clinic visit as the reason for non-participation. Men who were at the center for post-vasectomy semen analysis and/or receiving

treatment for infertility were excluded from the parent study. All participants completed a self-administered questionnaire. The questionnaire collected information about demographics, lifestyle factors, medical and fertility history. Information about potential confounding variables was also collected (e.g., age, abstinence time, smoking status, body-mass index (BMI), and race). A retrospective review of anonymized clinic records of non-participants, who met the same eligibility criteria as the study subjects, was previously performed and found that there were no differences between participants and non-participants in regards to age or semen parameters (Duty et al., 2005). Urine samples and semen samples were collected on the same day. As samples from the parent study had been used for other semen analysis research, eligibility for this analysis was based the availability of both a urine and semen sample for use from the biorepository. Of the men enrolled in the parent study (n=341), both a semen and urine sample of 181 men (53%) was available for this analysis. None of the men reported occupational exposure to pesticides or other agents. Informed consent forms were signed by all subjects prior to participation. The parent study was approved by the Harvard School of Public Health, the Massachusetts General Hospital Human Subjects Committees, and by the Office of Human Research at the George Washington University.

Semen Analysis

Measurement of the semen parameters have been previously described elsewhere (Hauser et al. 2003), and are briefly summarized below. Researchers asked the participants to abstain from ejaculation for 48 hours prior to providing a semen sample at the clinic via masturbation. Samples were liquefied at 37ºC for 20 minutes before analysis. Analysis of the samples took place at the MGH Andrology Laboratory. Andrologists were blinded

as to exposure status when the semen samples were analyzed. The volume, pH, color, and viscosity were determined for each sample. Sperm counts and percent motility were determined manually, and then measured by a computer-aided sperm analysis (CASA) using the Hamilton-Thorn Motility Analyzer (10HTM-IVO). A minimum of 200 sperm from 4 different fields were analyzed. CASA produced data on: 1) percentage of rapid and linear motile sperm; 2) average path velocity; 3) amplitude of lateral head displacement; and 4) linearity. Each sample was prepared on two slides for a morphological assessment. A Nikon microscope with an oil immersion 100x objective was used for this analysis (Nikon Company, Tokyo, Japan). Sperm were scored normal or abnormal using the rigorous Tygerberg Strict Criteria for morphology (<4% normal morphology) described by Kruger et al., 1988.

Disomy Analysis

Semen samples were stored in -80°C without cryoprotectant until FISH analysis was performed. Detection of sex chromosome disomy procedures have been outlined elsewhere (McAuliffe et al., 2012). A single investigator, blinded to exposure status, performed a Fluorescence in situ hybridization (FISH) analysis for the detection of disomy. The FISH procedure was carried out for three chromosomes: X, Y and 18 (autosomal control) to determine XX, YY, XY, and total sex-chromosome disomy in sperm nuclei. Sex chromosome disomy was the primary outcome of interest because sex chromosome disomy is the most common form of sperm aneuploidy, capable of producing X or Y disomic sperm suitable for fertilization and viable offspring. It is two times more frequent than disomy in the autosomes. A series of non-overlapping field images were taken for each prepared FISH slide. The images were taken using a

fluorescence microscope and scored using custom MATLAB software. The MATLAB software was designed to utilize scoring criteria for size and shape as reported by Baumgartner et al. (1999). Perry and colleagues (2011b) previously reported the procedures and validation of this semi-automated scoring method.

Urine Analysis

Urine samples were analyzed for metabolites cis/trans-2,2-(dichloro)-2-dimethylvinyl cyclopropane carboxylic acid (DCCA; which is a metabolite of permethrin, cypermethrin, and cyfluthrin); and 3-phenoxybenzoic acid (3PBA; a metabolite of multiple pyrethroid insecticides) using a small modification of the method of Baker et al, 2004. Briefly, the samples were spiked with isotopically labeled analogues of the target analytes to enable isotope dilution quantification. The target analytes were isolated using solid phase extraction, and the extracts were concentrated prior to analysis by high performance liquid chromatography-tandem mass spectrometry using an Agilent 6460 triple quadruple mass spectrometer (Santa Clara, CA) with Jetstream electrospray ionization. Quality control and blank samples were analyzed jointly with unknown samples to ensure method stability and robustness. The limits of detection were 0.1 ng/mL with relative standard deviations of <15%.

Statistical Analysis

Descriptive statistics were generated for demographic and semen parameters variables using frequency distributions or means and standard deviations. Semen parameters were dichotomized using the World Health Organization reference values for sperm concentration (<15 million sperm/mL) and motility (<32% motile sperm) (World Health

Organization, 2010) and the Tygerberg Strict Criteria for morphology (<4% normal morphology) (Kruger et al., 1988).

Insecticide urinary metabolite concentrations for 3PBA and DCCA, were specifically used as continuous as well as categorical measures (e.g., above or below the limit of detection (LOD) or quartiles). For metabolite values below the LOD, an imputed value equal to one-half the LOD was used. Descriptive statistics for pesticide metabolite levels (ng/mL) in urine were summarized as mean and standard deviation, geometric mean, median, and relevant percentiles. Creatinine concentrations and specific gravity were used to adjust for urine dilution. Zero-order correlations were used to examine the nature of the association between different urinary metabolites.

Furthermore, the association between different urinary metabolites and disomy (i.e., XX18, YY18, XY18, and total sex chromosome disomy) was examined using regression techniques. The adjusted and unadjusted regression parameters and confidence intervals have been reported. Poisson regression (SAS GENMOD procedure) was used to model the associations due to the large number of sperm being scored and the relatively low frequency of disomy (Appendix E). The Poisson regression was used to evaluate the contribution and significance between each disomy measure and the individual volume-based metabolite concentrations. Since the unit of analysis was the individual subject, the number of sperm scored and the number of disomic nuclei identified were summed for each subject.

Poisson models were fitted using a disomy measure (XX18, YY18, XY18, and total sex chromosome disomy) as the outcome variable (i.e., the natural logarithm of the number of sperm counted as the offset variable). The independent variable was the metabolite of

interest along with potential confounders such as age, race, smoking status, BMI, total sperm concentration, motility and morphology, and specific gravity as covariates in the adjusted analyses. Metabolites were entered into the model as: 1) dichotomous variables categorized as above and below the LOD for DCCA; and 2) variables categorized as quartiles for 3PBA. Incidence rate ratios (IRRs) and 95% confidence intervals were calculated for each model. Statistical analysis was performed using SAS version 9.3 (SAS Institute Inc, Cary, NC).

Results

Table 2.1 shows the mean and standard deviation of the demographic and semen parameter characteristics of the 181 study subjects. The men had an average age of 35.3 years and a mean BMI of 27.9 kg/m2. The majority of the men were white (84%) and non-Hispanic (8%). Most of the men (74%) had never smoked, with only 8% (n=14) current smokers. Of the 181 men, 10% (n=18) had sperm concentrations <15 million/mL, 21% (n=38) had <32% motile sperm, and 18% (n=33) had <4% normally shaped sperm. Table 2.2 provides a summary of the semen disomy results. A median of 5,945 sperm nuclei were scored per subject. The observed median percentages of XX18, YY18, XY18, and total disomy were 0.42, 0.37, 1.12, and 1.91 respectively.

Table 2.3 summarizes the mean and standard deviation for unadjusted urinary pyrethroid metabolite as well as the specific gravity (SG) and creatinine (CR) adjusted concentrations. Of the 181 samples, 80% (n=145) were above the LOD for 3PBA; only 9% samples (n=16) were above the LOD for DCCA. Although the percentage above LOD was higher for 3PBA, the median urinary levels were comparable for both

metabolites. The specific gravity and creatinine adjusted results were similar. Both pyrethroid metabolites were highly correlated (r=0.91).

Table 2.4 provides the mean percentage of each disomy outcome stratified by detectable levels of pyrethroid metabolites. No specific trends were observed between disomy values and exposure categories. Table 2.5 provides the Poisson regression model results, categorized by metabolite, for each disomy outcome. These Poisson regression results were adjusted for age, race, smoking status, BMI, total sperm concentration, motility and morphology, and specific gravity. Inverse associations were observed for XX18 (IRR_{Q4}=0.74, 95% CI: 0.67, 0.82), YY18 (IRR_{Q4}=0.94, 95% CI: 0.85, 1.04), XY (IRR_{Q4}=0.73, 95% CI: 0.69, 0.78), and total disomy (IRR_{Q4}=0.77, 95% CI: 0.74, 0.80) when the reference group (i.e., values < LOD) was compared to the highest 3PBA concentration levels (i.e., 4th quartile >0.83 ng/mL). Similar results were observed for DCCA when comparing rates of aneuploidy in those men with concentrations above the LOD to those men with concentrations below the LOD. The rates across the aneuploidy outcomes resulted in inverse risks, and no association was observed between DCCA and the different types of disomy.

Discussion

This epidemiological study examined the relationship between environmental PYR exposures and human sperm disomy outcomes. Results showed generally significant inverse rates of XX18, YY18, XY18, and total sex chromosome disomy by increasing levels of 3PBA and DCCA metabolites. Nonmonotonic dose-response trends were observed between the outcomes and 3PBA exposure categories, with an increase in disomy rates occurring in third exposure quartile and without an increase in fourth

exposure quartile. Men in the different exposure categories were similar in terms of their characteristics or semen parameters. Thus, it is unlikely that differences across exposure groups explain these nonmonotonic curves.

Registered PYR insecticides are metabolized in the body to create quantifiable 3PBA and DCCA metabolites. Unadjusted 95th percentile 3PBA urinary concentrations were lower in this study (2.27 ng/mL) when compared to the unadjusted urinary concentrations of males surveyed in the US general population reported for 2001-2002 (3.23 ng/mL) and 2009-2010 (6.50 ng/mL) (CDC, 2009 - Updated Tables 2015). In addition, unadjusted 95th percentile DCCA urinary concentrations in this study (2.65 ng/mL) were comparable to the urinary concentrations reported for 2001-2002 (2.44 ng/mL), but lower when compared to the most recent urinary concentrations for 2009-2010 (4.98 ng/mL) in the US general population (CDC, 2009 - Updated Tables 2015). We were able to evaluate relevant levels of environmental exposures.

Limited epidemiological data has been published about the relationship between pyrethroid pesticides and human sperm disomy outcomes. A prior study that evaluated the genotoxic effects of pyrethroids on male gametes, particularly sperm chromosome aberrations (i.e., in sex chromosomes and chromosome 18), reported that the frequency of sex chromosome disomy was significantly associated with fenvalerate exposure (Xia et al., 2004). Urinary concentrations of PYR metabolites (cis-DCCA and trans-DCCA) above the limit of detection (LOD) have been associated with increased rates of aneuploidy (Young et al., 2013). However, the findings for 3PBA metabolite and its association with aneuploidy were not consistent. A recent study found several associations between PYR pesticides and sperm disomy, suggesting that PYR pesticides

may be sperm aneugens (Radwan et al., 2015). Specifically, urinary levels of trans-DCCA (>50 percentile) were associated with XY disomy, while 3PBA urinary levels (≤50 and >50 percentile) were associated with XY disomy and total sex chromosome disomy.

Several human studies have evaluated the associations between pyrethroid pesticide exposure and sperm parameters, DNA damage and/or numerical chromosome aberrations (i.e., aneuploidy or diploidy) (Bian et al., 2004; Xia et al., 2004; Perry et al., 2007b; Meeker et al, 2008; Ji et al., 2011). Results from an occupational exposure study found associations between fenvalerate (a pyrethroid insecticide) and an increase in sperm DNA damage (Bian et al., 2004). Furthermore, urinary pyrethroid metabolites such as 3PBA and cis-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (CDCCA), have been associated with increased sperm DNA damage among men from the same parent study (Meeker et al., 2008). Ji et al. (2011) demonstrated an increase of DNA fragmentation in relation to 3PBA urinary concentrations among men from an infertile population.

The impact of pyrethroid exposure on sperm characteristics (including chromosomal damage) have been evaluated in animal studies for several specific PYR pesticides. Altered sperm characteristics due to cypermethrin exposure has also been reported in rats (Elbetieha et al., 2001). Research has also demonstrated that exposure to low doses of fenvalerate for 30 days reduces semen quality and sperm capacitation in adult mice (Shi et al., 2011). Furthermore, cyhalothrin (lambda) insecticide was found to induce a dose and time-dependent increase in the percentage of sperm abnormalities (i.e., structural and numerical chromosomal damage in primary spermatocytes after exposure) in mice (Abdel

Aziz and Abdel Rahem, 2010). Some of the abnormalities were in the form of fragment aneuploidy.

Moreover, androgen suppression in rats has been demonstrated to affect the expression of a number of proteins involved in cell division in male germ cells (Shi et al., 2011). Yuan et al. (2010) showed that fenvalerate and cypermethrin could reduce sperm motility in vitro and also evaluated if 3PBA directly or indirectly affected spermatogenesis via an interaction with androgens and/or their receptors. Their results showed that 3PBA did not reduce mature rat sperm motility directly at the concentrations and time periods tested (Yuan et al., 2010). Other studies have demonstrated that 3PBA has anti-androgenic activity in reporter gene assays (Sun et al., 2007; Du et al., 2010). Alterations in the expression of androgens and their potential impact to sex chromosome disomy requires further examination.

Pyrethroids are manufactured to kill target insects by affecting ion channel function, including chloride-permeable ion channels, voltage-gated calcium channels, and ligand-gated chloride channels. Specifically, their actions on the activation and inactivation of voltage-gated sodium channels (VGSCs) and neuronal excitability are well understood (US EPA, 2009b). Pyrethroids also have been reported to inhibit other types of ion channels (Shafer and Meyer, 2004). However, less is known about pyrethroid effects on other target organs such as the testis (Ray, 2001; Soderlund et al., 2002).

Because pyrethroids activate and inactivate the voltage-gated sodium channels, exposure to these chemicals may have an impact on the complexity of sperm regulation by affecting the functioning of the ion channels. Research has shown that VGSCs are

present in human sperm and have a role in supporting the regulation of mature sperm function (Pinto et al., 2009).

Although PYRs are widely used in the US, relatively little is known about the human reproductive health effects of environmental exposure to PYRs (ATSDR, 2003). The US EPA, through its Endocrine Disruptor Screening Program (EDSP), is currently screening chemicals for endocrine effects. Based upon the potential for exposure to each chemical, the Agency issued the first testing orders in 2009 for an initial list of 67 chemicals. From this initial list, 6 pyrethroids were selected for screening. Although there is a large body of literature on pyrethroid toxicology, not all biological effects, mechanisms, clinical signs and symptoms are completely understood, particularly in humans (US EPA, 2009b).

Prior studies have investigated PYR exposures and their association with endocrine disruption, hormone activities, and reproductive toxicity (Sun et al., 2007; Du et al., 2010; Ahmad et al., 2012). Studies using receptor mediated gene assays have shown that pyrethroids and their metabolites have the potential to disrupt the functions of nuclear hormone receptors (Du et al., 2010), resulting in adverse effects on the endocrine and reproductive systems in humans. Researchers have documented that mouse Sertoli cells exposure to pyrethroid pesticides affected estrogen receptors (Taylor et al., 2010). It is not known whether a similar effect can also occur in the human, or whether estrogenic EDCs may also have a similar effect.

In addition, the binding of natural pyrethrins and pyrethroids to steroid and xenobiotic-sensing nuclear receptors, such as the Pregnane X receptor (PXR), has also been reported (Yang et al., 2009). PXR expression is found primarily in the liver, with some expression

in the testis and embryonic tissue in humans (Timsit et al., 2007). In vivo (Zhang et al., 2008) and in vitro (Gill et al., 2011) effects of pyrethroids on pregnancy hormones have been reported; therefore, the potential role of pyrethroids and their effect on human reproductive toxicity cannot be disregarded.

It is uncertain how increased exposure to PYRs might be protective for sex chromosome disomy. Our results exhibited nonmonotonic dose-response relationships between exposure and effect. When nonmonotonic dose-responses occur, the effects observed at low doses cannot be predicted by the effects observed at high doses (Vandenberg et al., 2012). Low doses of a chemical could affect expression of a hormone receptor in the hypothalamus, an endpoint not examined in high-dose toxicology testing (Vandenberg et al., 2012). If cells in the male testis are exposed to low doses of estrogenic PYR pesticides, an effect on estrogen receptor expression may provide a molecular mechanism for disruption of normal spermatogenesis (Taylor et al., 2010).

PYR metabolites are limited as biomarkers of exposure. Studies have indicated that consumption of preformed PYRs may account for a noteworthy portion of urinary PYR concentrations (Sudakin, 2006; Starr et al., 2008; Sudakin and Stone, 2011). Urinary PYR metabolite levels may reflect exposure PYR parent compounds as well as other ambient metabolites. In addition, PYRs are generally rapidly metabolized by esterases (i.e., enzymes that split esters into an acid and an alcohol in a chemical reaction called hydrolysis). The detoxified metabolites are usually excreted from the body in a short period (Leng et al., 1990). Because PYRs are rapidly metabolized and the spermatogenic cycle lasts from 75-90 days, a single urinary measurement is not likely to be a robust exposure measurement and these analyses assumed that this urinary measurement is

reflective of a longer term exposure. Also, there is the need to evaluate the effects and impact on spermatogenesis caused by the exposure to preformed degradation products versus exposure to parent compounds. Nonetheless, urinary pesticide metabolite analyses continue to be extensively used in biomonitoring studies due to their relatively low costs and their utility for interpreting health outcomes of interest. Despite these limitations, the current study provides instrumental information about cumulative exposure (i.e., numerous PYR active ingredients via multiple exposure routes and pathways) to the PYR pesticide class.

The men in this study population were recruited from a fertility clinic as opposed to the general population. There is no evidence that this recruitment setting limits the external validity of our results because there is no indication that our participants differ in their response to PYR exposures from men in the general population. About 50% of the men in our study population had normal semen motility, concentration and morphology; thus, increasing the generalizability of our results. Couples attending fertility clinics represent couples with male factor infertility, female factor infertility, or both. In addition, we collected extensive information on potential confounders that were modeled simultaneously with the exposure. In the current study, disomy frequencies are higher than other frequency distributions previously reported (Egozcue et al., 1997; Templado et al., 2005). However, those studies did not include men seeking fertility consultation. A validated semi-automated method for disomy frequency determination was used in this study. The semiautomated method allowed for an objective counting of disomic sperm in a large number of samples. (Perry et al., 2007a, 2011a).

Conclusion

This epidemiologic study examined the relationship between environmental PYR exposures and human sperm disomy outcomes by conducting multiple analyses adjusted for potential confounders. Findings suggest that inverse disomy rates were associated with PYR metabolites. It is uncertain how increased exposure to EDCs, such as PYR pesticides, might be protective for sperm chromosome disomy. These findings are not consistent with other results reported in the scientific literature. Nonmonotonic dose-response relationships were observed between the outcomes and 3PBA exposure categories. There are multiple mechanisms by which EDCs can modulate endocrine systems and potentially cause reproductive effects. Questions remain as to how environmental exposures to PYR pesticides affect sperm sex chromosomal abnormalities. The results of this study were used to inform additional analyses (see Chapter 4).

Table 2.1. Characteristics of MGH Men.

Table 2.1: Characteristics of MGH men (n=181).	
Variable	**Mean ± SD**
Age	35.3 ± 5.1
BMI (kg/m^2)	27.9 ± 4.7
Race (n=2 missing)	**N (%)**
White	152 (84.0)
Black	5 (2.7)
Other	24 (13.3)
Hispanic ethnicity	
No	168 (92.8)
Yes	13 (7.2)
Semen Concentration	
<15 million/mL	18 (9.9)
Semen Morphology	
<4% normal	33 (18.2)
Semen Motility	
<50% motile	38 (20.9)
Abstinence time	
<=2 days	37 (20.4)
3-4 days	87 (48.1)
>=5 days	57 (31.5)
Smoking (n=2 missing)	
No	132 (73.7)
Current smoker	14 (7.8)
Former smoker	33 (18.5)

Table 2.2. Number of Sperm Nuclei Scored and Percent Disomy.

Table 2.2: Number of sperm nuclei scored and percent disomy of men seeking infertility evaluation (n=181).				
Variable	**Mean ± SD**	**Median**	**25th**	**75th**
Nuclei (n)	7026 ± 4708	5945	3380	9976
% XX18	0.42 ± 0.41	0.27	0.16	0.52
% YY18	0.37 ± 0.32	0.29	0.19	0.46
% XY18	1.12 ± 0.84	0.90	0.56	1.48
Total Disomy %	1.91 ± 1.28	1.55	1.10	2.47

Table 2.3. Distribution of Pesticide Metabolite Levels in Urine.

Table 2.3: Distribution of pesticide metabolite levels in urine of men (n=181).							
Metabolite* (ng/mL)	**Mean ± SD**	**Percentile**					**Range**
		25th	**50th**	**75th**	**90th**	**95th**	
Unadjusted							
3PBA	0.92 ± 1.51	0.52	0.64	0.87	1.45	2.27	0.05-15.18
DCCA	0.61 ± 2.16	0.05	0.05	0.10	0.94	2.65	0.0012-16.99
SG-adjusted							
3PBA (n=152)	1.22 ± 2.38	0.45	0.73	1.27	2.27	2.90	0.03-27.83
DCCA (n=152)	0.74 ± 2.93	0.05	0.08	0.18	0.90	2.48	0.0011-31.15
CR-adjusted							
3PBA (n=169)	1.06 ± 3.64	0.26	0.47	1.00	1.72	2.70	0.02-46.56
DCCA (n=169)	0.75 ± 4.22	0.03	0.06	0.14	0.70	2.13	0.00043-52.12

*3PBA & DCCA LOD=0.1.

Table 2.4. Aneuploidy Mean ± SD by Detection of Pyrethroid Metabolite Levels.

Table 2.4: Aneuploidy Mean ± SD by detection of pyrethroid metabolite levels (n=181).				
Metabolite[a]	**%XX18**	**%YY18**	**%XY18**	**% Total Disomy**
3PBA				
Q1	0.39 ± 0.38	0.40 ± 0.45	1.22 ± 1.14	2.01 ± 1.78
Q2	0.44 ± 0.32	0.32 ± 0.18	1.12 ± 0.72	1.88 ± 0.96
Q3	0.37 ± 0.34	0.35 ± 0.21	1.09 ± 0.77	1.81 ± 1.13
Q4	0.47 ± 0.56	0.41 ± 0.38	1.09 ± 0.76	1.97 ± 1.29
DCCA				
G1	0.43 ± 0.42	0.37 ± 0.30	1.14 ± 0.88	1.94 ± 1.34
G2	0.40 ± 0.39	0.35 ± 0.35	1.09 ± 0.71	1.84 ± 1.11

[a] 3PBA & DCCA LOD=0.1. 3PBA Exposure Quartiles: Q1=X≤LOD (n=36), Q2=0.10<X≤0.61 ng/mL (n=50), Q3=0.61<X≤0.83 ng/mL (n=46), Q4=X>0.83 ng/mL (n=49). DCCA Exposure Group: G1=X≤LOD (n=136); G2=X>0.10 ng/mL (n=45).

Table 2.5. Adjusted IRRs for each Disomy Outcome by Pyrethroid Metabolite.

Table 2.5: Adjusted IRRs (95% CI) for XX, YY, XY, and total sex-chromosome disomy by pyrethroid metabolite of men seeking infertility evaluation (n=181).

Metabolite[a]	Adjusted IRRs[b]			
	XX18	YY18	XY18	Total Disomy
3PBA				
Q1	1.00	1.00	1.00	1.00
Q2	0.89 (0.81, 0.98)	1.25 (1.14, 1.38)	0.77 (0.73, 0.81)	0.87 (0.84, 0.91)
Q3	1.04 (0.95, 1.13)	0.96 (0.87, 1.05)	0.93 (0.89, 0.98)	0.96 (0.92, 0.99)
Q4	0.74 (0.67, 0.82)	0.94 (0.85, 1.04)	0.73 (0.69, 0.78)	0.77 (0.74, 0.80)
DCCA				
G1	1.00	1.00	1.00	1.00
G2	0.86 (0.90, 0.92)	1.03 (0.95, 1.10)	0.90 (0.86, 0.94)	0.91 (0.88, 0.94)

[a] 3PBA & DCCA LOD=0.1. 3PBA Exposure Quartiles: Q1=X≤LOD (n=36), Q2=0.10<X≤0.61 ng/mL (n=50), Q3=0.61<X≤0.83 ng/mL (n=46), Q4=X>0.83 ng/mL (n=49). DCCA Exposure Group: G1=X≤LOD (n=136); G2=X>0.10 ng/mL (n=45).
[b] IRRs were adjusted for specific gravity, age, race, BMI, smoking, total concentration, motility and morphology.

Chapter 3: Dialkyl Phosphate Urinary Metabolites and Sperm Chromosomal Abnormalities

Introduction

The impacts of environmental endocrine disruptors on male reproductive health has received heightened research attention in recent years (Diamanti-Kandarakis et al., 2009; Woodruff, 2011; Zoeller et al., 2012; WHO, 2013). Each year more than 2 million couples in the US who want to have children are infertile, and over 2 million conceptions are lost before the twentieth week of gestation (ACOG, 2002; CDC, 2013). Most aneuploid conceptuses perish in utero; up to 50% of all spontaneous abortions are thought to be related to pre-existing chromosomal abnormalities (Jacobs, 1992: Lebedev et al., 2004). Because many chromosomal abnormalities come from the father's sperm, particularly for the sex chromosomes (X and Y), researchers have attempted to understand the paternal role in sex chromosome aneuploidy (Hassold and Hunt, 2001; Martin et al., 1991). Aneuploidy occurs when chromosome pairs fail to separate properly during cell division. In germ cells, errors in chromosome segregation during meiosis (I or II) result in imbalances in chromosome numbers; however, the exact causes of non-disjunction are unknown. Disomy is the most frequent aneuploidy observed in human sperm.

Children with sex chromosomal abnormalities (e.g., characterized in Kleinfelter and Turner syndrome), may have reproductive disorders, behavioral and/or intellectual difficulties when compared to their siblings (Martin, 2006; Boyd et al., 2011). Evidence from European birth defect registries suggests that the prevalence of chromosomal abnormalities in infants (during the first 28 days after birth) increased between 1967-

1988 (Morris et al., 2008). Because there was no observed increase in maternally-derived chromosomal abnormalities, underlying environmental causes affecting spermatogenesis are suspected (Morris et al., 2008). Comparable birth defect data for the US are not available.

Much concern has been raised about pesticides being potential endocrine disrupting chemicals (EDCs). EDCs can modulate the endocrine system and potentially cause adverse effects (Sharpe, 2009; Woodruff, 2011; Zoeller et al., 2012; WHO, 2013; NAS, 2014). Humans are exposed to EDCs through multiple routes of exposure (oral, dermal and inhalation) and pathways, including their diet (direct, indirect), environment (water, soil, air), and occupation (Tyler et al., 2000; Jørgensen et al., 2006; McKinlay et al., 2008a, 2008b; Mnif et al., 2011). Because organophosphate (OPs) insecticides accounted for a large share of all US insecticide use, they were the first group of pesticides to be reviewed under the Food Quality Protection Act (FQPA) of 1996. In 1999, the US EPA determined a common mechanism of action based on their ability to bind to and phosphorylate the enzyme acetylcholinesterase in both the central (brain) and peripheral nervous systems (US EPA, 1999). OPs are used in agriculture, recreational and commercial areas, and public pest control programs, accounting for 35% of the total US insecticide usage (US EPA, 2011). Urinary metabolites of OPs, such as dialkyl phosphates (DAPs), have been measured in a substantial proportion of the general population (Barr et al., 2004; CDC, 2009). OPs have been associated with effects on thyroid hormone levels (Lacasaña et al., 2010), decreased semen volume and sperm count (Yucra et al., 2008; Recio-Vega et al., 2008), lower sperm concentration (Perry et al., 2007b), abnormal morphology and decreased sperm motility (Hossain et al., 2010), DNA

damage/fragmentation in sperm (Meeker et al., 2004; Muñiz et al., 2008; Atherton et al., 2009), and sperm chromatin structure alteration (Sanchez-Pena et al., 2004). However, limited information has been published about associations between OPs and sperm abnormalities (Padungton et al., 1999; Recio et al., 2001).

Toxicants may adversely affect germ cell DNA integrity (Mruk and Cheng, 2011); however, the exact causes of aneuploidy and the specific windows in which exposures impact the spermatogenic cycle are not well known (Herrera et al., 2008; Axelsson et al., 2010; Ashton Acton, 2013). This study investigated environmental exposures to OPs and their association with altered frequency of disomy among adult men.

Materials and Methods

Study Subjects

Study subjects were men from a parent study assessing the impact of environmental exposures on semen quality. The parent study has been described elsewhere (Hauser et al., 2003). Briefly, eligible participants were men aged 20-54 from couples seeking infertility evaluation at Massachusetts General Hospital (MGH) Fertility Center between January 2000 and May 2003. Sixty-five percent of eligible men agreed to participate; those declining participation cited lack of time during their clinic visit. Exclusion criteria included men who were at the center for post-vasectomy semen analysis and/or receiving treatment for infertility. None of the men reported occupational exposure to pesticides or other agents. All men completed a self-administered questionnaire that collected demographic, lifestyle factors, medical and fertility history information, and provided urine and semen samples. Eligibility for this analysis was based on the availability of

both a urine and semen sample from the biorepository. Of the men enrolled in the parent study (n=341), a semen sample was available for 159 men (47%). Informed consent forms were signed by all subjects prior to participation. The parent study was approved by the Harvard School of Public Health, the Massachusetts General Hospital Human Subjects Committees, and by the Office of Human Research at the George Washington University.

Semen Analysis

Measurement of the semen parameters have been previously described (Hauser et al. 2003). Researchers asked the participants to abstain from ejaculation for 48 hours prior to providing a semen sample at the clinic via masturbation. Samples were liquefied at 37ºC for 20 minutes before analysis. Analysis of the samples took place at the MGH Andrology Laboratory. Andrologists were blind as to exposure status. The volume, pH, color, and viscosity were also determined for each semen sample. Sperm counts and percent motility were determined manually and then measured by computer-aided sperm analysis (CASA) using the Hamilton-Thorn Motility Analyzer (10HTM-IVO). A minimum of 200 sperm from 4 different fields were analyzed. A Nikon microscope with an oil immersion 100x objective was used for this analysis (Nikon Company, Tokyo, Japan). Sperm were scored normal or abnormal using the strict criteria reported by Kruger et al., 1988.

Disomy Analysis

Semen samples were stored in -80°C without cryoprotectant until FISH analysis was performed. The procedures for the detection of sex chromosome disomy have been

described elsewhere (McAuliffe et al., 2012). A single investigator, blinded to exposure status, performed Fluorescence *in situ* hybridization (FISH) analysis for the detection of sex chromosome disomy, as the primary outcome of interest. Sex chromosome disomy is the most frequent form of sperm aneuploidy, occurring twice as frequently as disomy in the autosomes and resulting in viable offspring (i.e., disomic sperm for X or Y are capable of fertilization). The FISH procedure was carried out for three chromosomes of interest: X, Y and 18 (autosomal control) to determine XX18, YY18, XY18 and total sex chromosome disomy in sperm nuclei. A series of non-overlapping field images were taken for each FISH slide using a fluorescence microscope and scored using custom MATLAB (Mathworks Inc., Natick, MA) software. The software was designed to utilize scoring algorithms based on criteria for size and shape as reported by Baumgartner et al. (1999). Details of the sperm FISH control procedures and validation of the semi-automated scoring method have been previously reported (Perry et al., 2007a, 2011a).

Urine Analysis

Urinary dialkylphosphate (DAP) metabolites were used to estimate human exposure to OP pesticides. Concentrations of six DAP metabolites (i.e., dimethylphosphate (DMP); dimethylthiophosphate (DMTP); dimethyldithiophosphate (DMDTP); diethylphosphate (DEP); diethylthiophosphate (DETP); and diethyldithiophosphate (DEDTP)) were analyzed in urine specimens, using gas chromatography (GC) coupled with flame photometric detection (FPD) with isotopic dilution quantification. This method is reported elsewhere (Prapamontol et al., 2014). Briefly, an aliquot of 5.0 mL of urine was pipetted and mixed into 2 g of NaCl in a15 mL glass test tube and 1.0 ml of 6N solution of Hydrochloric acid (HCl). The sample was extracted with acetone (5.0 mL), shaken

and centrifuged. The extract was evaporated almost to dryness before adding acetonitrile. The dried residue was re-dissolved three times with acetonitrile; these solutions were then combined with K_2CO_3 and PFBBr. The sample was incubated and water was added after derivatization. The derivatives were extracted twice in hexane; these hexane layers were then combined. The solution was evaporated under a nitrogen stream in a water bath with set temperatures (35-40°C). The dried residues were dissolved with toluene for injection into the GC-FPD.

The DAP metabolites are divided in two main groups: the dimethyl alkylphosphates (or DMAPs), and the diethyl alkylphosphates (or DEAPs). Measuring ΣDAP, ΣDEAP and ΣDMAP is a common approach to quantifying total exposure to organophosphates (Barr et al., 2004). The methyl-containing metabolites (or DMAPs) are derived from O,O-dimethyl–substituted OP pesticides such as azinphos-methyl, dimethoate, malathion, phosmet, among others (Appendix A). During metabolism, the phosphoric group of the parent OP undergoes hydrolysis to become DMP, DMTP, and/or DMDTP. The ethyl-containing metabolites (or DEAPs; including DEP, DETP, and/or DEDTP) are derived from O,O-ethyl–substituted OP pesticides such as chlorpyrifos, diazinon, parathion, among others (Appendix A). Most OP-ethyl parent pesticides are metabolized into DEP and DETP metabolites.

The limit of detection (LOD) for each analyte was estimated by spiking pooled urine samples with standard solutions (from serial dilution of the lowest standard calibration solution) to obtain a concentration providing a signal-to-noise ratio (S/N) of ≥3. The LODs were 0.6 ng/mL (DMP), 0.2 ng/mL (DMTP), 0.2 ng/mL (DEP), and 0.1 ng/mL (DETP, DMDTP, DEDTP). Total organophosphate metabolite concentration (ΣDAP),

total diethyl alkylphosphate (ΣDEAP or sum of DEP, DETP and DEDTP metabolites), and total dimethyl alkylphosphate (ΣDMAP or sum of DMP, DMTP and DMDTP metabolites) were calculated by first dividing each metabolite concentration by its molecular weight (154, 170, 186, 126, 132, 158 for DEP, DETP, DEDTP, DMP, DMTP, and DMDTP, respectively). These transformed metabolite concentrations were then summed and multiplied by 1,000 to obtain units of nmol/mL. Urine samples specific gravity was measured using a handheld refractometer (National Instrument Company, Inc., Baltimore, MD, USA). Creatinine was measured photometrically using kinetic colorimetric assay technology with a Hitachi 911 automated chemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA).

Statistical Analysis

Descriptive statistics were generated for demographic and semen parameter variables. Semen parameters were dichotomized using the most recent World Health Organization (WHO) reference values for sperm concentration (<15 million sperm/mL) and motility (<32% motile sperm), and the Tygerberg Strict Criteria for morphology (<4% normal morphology) (Kruger et al., 1988; World Health Organization, 2010). Urinary metabolite concentrations were used as both continuous and categorical measures. For metabolite values below the LOD, an imputed value equal to one-half the LOD was used. Descriptive statistics for pesticide metabolite levels (μg/L) in urine were summarized. Creatinine and specific gravity adjusted concentrations as well as volume-based (unadjusted) values were calculated. Although creatinine concentrations are commonly used to adjust for urine dilution, studies have reported that creatinine levels vary by individual factors such as sex (Bjornsson et al., 1979; Turner et al., 1975); age (Edwards

et al., 1959; Fuller et al., 1982); diet (Lykken et al., 1980); decreasing muscle mass (Alessio et al., 1985; Driver et al., 1980); and time of day or seasonality (Freeman et al., 1995; O'Rourke et al., 2000). Therefore, creatinine concentrations may not be suitable to adjust for urine dilution. Specific gravity is considered to be more appropriate and was used to adjust urine metabolite concentrations (Barr et al., 2005). Because the urinary concentrations were not normally distributed, these data were log transformed. Pearson correlations were examined to explore the nature of the association between individual urinary DAP metabolites as well as with the total sum of specific metabolite products (i.e., ΣDEAP, ΣDMAP, and ΣDAP).

Due to the large number of sperm being scored and the relatively low frequency of disomy, the association between each specific volume-based urinary metabolite concentration and the disomy measures (i.e., XX18, YY18, XY18, and total sex chromosome disomy) were modeled using Poisson regression (SAS GENMOD procedure) in unadjusted and adjusted models (Appendix E). All outcome variables were examined, and all displayed a classic Poisson distribution shape. For each subject, the number of sperm scored and the number of disomic nuclei were summed. The individual subject was treated as the unit of analysis. The number of sperm counted was standardized across subjects using the offset variable of the natural logarithm of the number of sperm scored.

Poisson models were fitted using a disomy measure as the outcome variable (i.e., as a count of disomic cells for XX18, YY18, XY18, and total sex chromosome disomy) and the metabolite of interest as the independent variable. Age, body-mass index (BMI), motility, morphology, log of sperm concentration and specific gravity were included as

continuous covariates. Smoking (never, former, and current) and race (white, black, or other) were included as categorical covariates. Because the distribution of sperm concentration is often non-normal and positively skewed (Berman et al., 1996), a log transformation of sperm concentration was used. Metabolites were categorized as quartiles for most metabolites. Incidence rate ratios (IRRs) and 95% confidence intervals were calculated for each model. In addition, the exposure variable was included in the model as an ordinal variable to test for trend. Because the urine samples may have been too concentrated or too diluted to provide valid results, a sensitivity analysis was performed excluding individuals (n=9) with creatinine concentrations >300 mg/dL or <30 mg/dL or specific gravity >1.03 or <1.01 (n=29). A sensitivity analysis was also performed excluding those individuals (n=8) with fewer than 1,000 nuclei scored, as too few nuclei scored could impact the disomy estimates. Because individual DAP metabolites are correlated among themselves and constitute a portion of ∑DAPs, models were adjusted for ∑DAPs (i.e., total sum of all available DAP metabolites) to account for exposure interrelationships. Equivalent results were obtained and ∑DAPs was removed from the final adjusted models. Statistical analysis was performed using SAS version 9.3 (SAS Institute Inc., Cary, NC).

Results

Table 3.1 shows the demographic and semen parameter characteristics of the study subjects (n=159). The men had an average age of 35 years and a mean BMI of 28 kg/m^2. The majority of the men were white (86%) and non-Hispanic (94%). Most men (74%) had never smoked with only 7% current smokers. Of the 159 men, 10% (n = 16) had sperm concentrations <15 million/mL, 21% (n = 33) had <32% motile sperm, and 18% (n

= 28) had <4% normally shaped sperm based on the most recent WHO reference values for sperm concentration and the Tygerberg Strict Criteria for morphology. A median of 6848 sperm nuclei were scored per subject (Table 3.2). The observed median percentages of XX18, YY18, XY18, and total disomy were 0.4, 0.4, 1.1 and 1.9, respectively. The %XY18 median was 3 times higher than %XX18 and %YY18 medians.

Table 3.3 summarizes the unadjusted urinary OP metabolites as well as the specific gravity and creatinine adjusted concentrations. About 57% of the urinary samples were above the LOD for most metabolites. DMTP was the most frequently detected metabolite (> 80% of the samples). DMP metabolites were detected at the highest concentration levels. Although the percentage above the LOD was higher for DMTP, the median levels were slightly higher for DMP and DMDTP. The specific gravity and creatinine adjusted results were similar. All three DMAP metabolites (r = 0.48-0.57) and DEAP metabolites (r = 0.20-0.54) were weakly to moderately correlated. Total DAPs and DMAPs were strongly correlated (r = 0.97), and there were moderate correlations between Total DAPs and DEAPs (r = 0.73) and between DEAPs and DMAPs (r = 0.58).

Table 3.4 provides the mean percentage of each of the evaluated disomies as well as total disomy stratified by detectable levels of OP metabolites. Men in the highest quartile had consistently higher values for %XX18 and total disomy. Similar disomy values were observed in other exposure quartiles.

Table 3.5 shows the results of the Poisson regression models for each outcome adjusted for specific gravity, age, race, BMI, smoking, total concentration, motility, and morphology when categorizing metabolites into exposure quartiles. Urinary DMTP

levels above the lowest quartile were significantly associated with increased rates of XX18, YY18, XY18, and total sex chromosome disomy. The highest significant association was observed between the third exposure quartile of DMTP (2.21-6.47 ng/mL) and XX18 , with a 52% increase in the incidence rate ratio (IRR_{Q3} = 1.52; 95% CI: 1.36, 1.69) when compared to the reference group (i.e., first quartile). Significantly increased disomy rates were also observed for men in different exposure quartiles of individual DMDTP, DEP and DETP metabolites. Specifically, increased rates were observed for DMDTP and XX18 (IRR_{Q3}=1.24; 95% CI: 1.14, 1.36), YY18 (IRR_{Q2}=1.26; 95% CI: 1.15, 1.38), XY18 (IRR_{Q3}=1.28; 95% CI: 1.22, 1.35) and total sex chromosome disomy (IRR_{Q3}=1.23; 95% CI: 1.18, 1.28). Increased rates were observed between DEP and XX18 (IRR_{Q2}=1.23; 95% CI: 1.12, 1.35), YY18 (IRR_{Q3}=1.45; 95% CI: 1.33, 1.59), XY18 (IRR_{Q3}=1.09; 95% CI: 1.04, 1.15) and total sex chromosome disomy (IRR_{Q3}=1.16; 95% CI: 1.11, 1.21). Results also showed that DETP was associated with increased rates of XX18, YY18 and total disomy but was inversely associated with XY18. Inverse associations were observed for DMP and XX18, XY18 and total sex chromosome disomy. For DEDTP, only 16 men were above the LOD; inverse associations were observed for YY18, XY18, and total sex chromosome disomy. Adjusted IRRs for XX18, YY18, XY18, and total sex chromosome disomy by ∑DAPs showed inverse associations when compared to the reference group. Additionally, adjusted IRRs for each disomy outcome by ∑DMAPs and ∑DEAPs were also calculated (Appendix B). These results were similar to those observed for ∑DAPs. Therefore, these results (∑DMAPs and ∑DEAPs) are not discussed further in this study.

Because individual DAP metabolites are subsets of the total DAPs and are therefore highly correlated, adjusted models that controlled for total DAPs were also run. Similar IRRs were obtained.

Linear tests for trend were examined in adjusted models (Table 3.5). Dose-response curves appeared nonmonotonic, with increase in disomy rates mainly occurring between the second and third exposure quartiles. For example, an increase in the magnitude of total disomy rates between the second and third exposure quartiles of DMDTP was observed; however, after the third DMTP exposure quartile, the magnitude of the XX18 disomy rate decreased (p-value for trend=0.0001). Even though significant p-values for trend were achieved, nonmonotonic patterns were widely observed across disomy types. Sensitivity analyses, excluding 9 individuals with creatinine concentrations >300 mg/dL or <30 mg/dL, and 29 individuals with specific gravity concentrations >1.03 or <1.01, showed similar results (data not shown). A sensitivity analysis with total nuclei scored < 1,000 was also conducted, as disomy estimates can be impacted by too few nuclei scored. In the reanalysis, eight men were excluded and the results remained essentially unchanged.

Discussion

This is the first epidemiologic study of this size to examine the relationship between environmental OP exposures and human sperm disomy outcomes. Results showed significant increased rates of XX18, YY18, XY18, and total sex chromosome disomy (7-52%) by increasing levels of DMTP, DMDTP, DEP and DETP metabolites after adjusting for potential confounders (i.e., age, race, BMI, smoking, specific gravity, total sperm concentration, motility, and morphology). Even though other lifestyle factors may

affect chromosomal abnormalities, only risk factors with known associations with pesticide exposure and sperm disomy outcomes were included in the adjusted models. A significant inverse association was observed in adjusted models between DMP and XX18, XY18 and total disomy. The relationship between the disomy outcomes and the individual DAP metabolites as well as the relationship with ∑DAPs were examined to identify their underlying differences and distinctive associations. Adjusted IRRs for XX18, YY18, XY18, and total sex-chromosome disomy by ∑DAPs showed inverse associations when compared to the reference group. These results suggest that aggregating all six DAP metabolites into ∑DAPs may conceal the independent effects of each metabolite. ∑DAPs showed mostly inverse associations across disomy types.

Nonmonotonic dose-response trends were observed between the outcome and exposure categories, with most of the increase in disomy rates occurring between the second and third exposure quartiles and without substantial additional increases between the third and fourth exposure quartile. Because men in the reference group were not significantly different from men in the highest exposure quartile in terms of their demographic and lifestyle characteristics or semen parameters, it is unlikely that differences across exposure groups explain these nonmonotonic curves.

Unadjusted DAP urinary concentrations (males, 95th percentile) were slightly higher in the current study (0.39-39 ng/mL) than the levels reported in the U.S. general population for 2001-2002 (0.78-31 ng/mL) and 2007-2008 (<LOD-36 ng/mL), as reported in the Fourth National Report on Human Exposure to Environmental Chemicals (CDC, 2009 - Updated Tables 2015). In our study, the metabolites detected at highest concentrations

were DMP, DMTP and DEP. Similarly studies have reported DMTP as the metabolite detected at highest concentration, followed by DMDTP and DEP (Recio et al., 2001).

Limited epidemiological information has been published about the effects of organophosphate pesticides and their association with sex chromosome disomy (Padungtod et al., 1999; Recio et al., 2001). Increased frequency of total sperm aneuploidies (0.30%) were observed among Chinese pesticide workers manufacturing methyl parathion, ethyl parathion, and metamidophos, compared with the controls (0.19%) (Padungtod et al., 1999). Also, an increased prevalence of sperm aneuploidies in chromosomes X, Y, and 18 was also observed in these workers. A cross-sectional study among agricultural workers also found similar significant associations between frequencies of sperm aneuploidy and organophosphate urinary metabolites (Recio et al., 2001). The most frequent aneuploidy observed was the lack of a sex chromosome or sex null (0.19%), followed by XY18 (0.15%). Increased relative risks were observed between DEP (2.59, 95% CI: 1.59, 2.71) and DETP (1.68, 95%: 1.13, 2.81) and the sex null during the spraying season. Total aneuploidies were significantly higher (72%) during the pesticide spraying season when compared to before the spraying season (59%).

Several studies have reported that OP pesticide exposures were significantly associated with decreased semen volume and decreased sperm count (Yucra et al., 2008; Recio-Vega et al., 2008), lower sperm concentration (Padungtod et al., 2000; Perry et al., 2007b), sperm chromatin alteration (Sanchéz-Peña et al., 2004), increased luteinizing hormone and decreased testosterone (Padungtod et al., 1998), higher abnormal morphology and decreased sperm motility (Hossain et al., 2010). Some OPs such as

parathion and methylparathion are structurally similar to various hormones, including estrogens and may interact with hormone receptors and/or gene transcription. Perry et al. (2011b) reported that high levels of urinary DMP metabolites were associated with sperm concentrations and total motility below the median population levels among Chinese men. A cross-sectional study conducted among Mexican farmers evaluated the effects of OP toxicity on semen quality and DNA integrity. Two OP exposure indexes were created: at the month of sampling (representing the exposure to spermatids-spermatozoa) and during 3 months before sampling (representing cells at one spermatogenic cycle). In this study, dose-response relationships were observed between OP exposure and sperm quality parameters for both exposure indexes. This study suggested that cells at all stages of spermatogenesis are affected by OP chemicals (Perez-Herrera et al., 2008).

The commonly known mechanism of action of OP toxicity involves the inhibition of the enzyme acetylcholinesterase (AChE) which leads to neurotoxicity in the central and/or peripheral nervous system (US EPA, 1999). It is likely that OP pesticides also act through noncholinergic mechanisms (i.e., without inhibition of acetylcholinesterase) at low environmental doses (Dam et al., 2003). Various pesticides have been found to bind and alter the function of hormone receptors, alter the synthesis or clearance of endogenous hormones, interact with various neurotransmitter systems, and cause adverse effects by other mechanisms (Stoker et al., 2000). With respect to the OPs, less is known about OP effects on other target organs such as the testis. Because spermatogenesis is a highly complex process dependent upon optimal conditions to occur correctly (Cheng and Mruk, 2009, 2010), OP secondary mechanisms that are not associated with the cholinergic system, including inhibitory mechanisms of androgenic activity mediated by

hormones, may lead to endocrine disrupting effects (Elersek and Filipic, 2011; Kang et al., 2004).

OPs prevent thyroid hormone-receptor binding and increase the expression of estrogen responsive genes (McKinlay et al., 2008b). Toxicology studies have linked OPs with effects on steroid and thyroid hormones in rats (Jeong et al., 2006), anti-androgenic activity in rats (Kang et al., 2004), and increased spermatids apoptosis during spermatogonial proliferation in mice (Masoud et al., 2003). Severe spermatogenesis disruption (specifically affecting the pituitary gonadotrophins) was shown with increasing doses of quinalphos in male rats (Sarkar et al., 2000). Furthermore, increased follicle stimulating hormone (FSH) levels have been observed in rats exposed to chlorpyrifos suggesting adverse effects on their reproductive system (Sai et al., 2014). A study in adult male mice measured FSH and luteinizing hormone (LH) concentrations and reported diazinon effects on testis structure and sex hormones levels (Fattahi et al., 2009). Significant reductions were also observed in the diameter and weight of testes, Leydig and Sertoli cells, sperm count, and testosterone concentration. FSH and LH triggered testosterone production potentially altering spermatogenesis mechanisms. Similarly, Ngoula et al. (2007) observed spermatogenesis inhibition and Leydig cells density reduction after male rats were treated with pirimiphos-methyl. It appears that OP pesticides disrupt male hormones; however, it is still uncertain the relationship between OP exposure, hormone signaling and cell division.

Epidemiological studies among non-occupationally exposed adult males have found associations between OP exposure and hormones serum levels (i.e., thyrotropin (TSH) and triiodothyronine (T4)) (Meeker et al., 2006). Recio et al. (2005) found inverse

associations between FSH levels and DMTP and DMDTP, and between DMTP levels and LH among Mexican agricultural workers. Another investigation among Peruvian pesticide applicators reported no significant associations between DAPs and hormone levels (Yucra et al., 2008). An inverse association between DAP and total thyroxine (T3) was also reported among floricultural workers living in Mexico (Lacasaña et al., 2010). Nonetheless, an increased association between total DAPs and TSH and T4 levels was also demonstrated among the same group of men. Both inverse and increased associations were observed between hormones (i.e., inhibin B, FSH, LH) and DAP metabolites (Blanco-Muñoz et al., 2010). These epidemiological findings are consistent with discussions of potential low-dose effect mechanisms responsible for generating human disorders (Vandenberg et al., 2012).

The endocrine system is likely to respond to very low hormone concentrations allowing a vast number of hormonally active molecules to co-occur (Welshons et al., 2003). Low doses of a chemical could affect expression of a hormone receptor in the hypothalamus, an endpoint not examined in high-dose toxicology testing (Vandenberg et al., 2012). It is uncertain how increased exposure to OPs might be protective for sex-chromosome disomy. Our results exhibited nonmonotonic dose-response relationships between exposure and effect. When nonmonotonic dose-responses occur, the effects observed at low doses cannot be predicted by the effects observed at high doses (Vandenberg et al., 2012). Scientists have previously stated that as the dose decreases and the effect size decreases, the number of animals or individuals needed to achieve the power to detect a significant effect would have to increase substantially (vom Saal et al., 1998). Even though active compounds may induce significant biological effects at extremely low

concentrations and researchers may be unable to detect small magnitudes of effect (Vandenberg et al., 2012), this study was able to detect significant associations between OP exposures and disomy.

Detailed exposure assessments that include time-specific exposure are needed to determine when the most critical exposures windows to the spermatogenic process are likely to occur. Spot urine samples are unlikely to reflect cumulative pesticide exposures, particularly when the parent compounds are rapidly metabolized (Martenies and Perry, 2013). OP metabolites measured in a single urine sample may not accurately reflect cumulative exposure over longer periods due to urine volume variability and the concentrations of endogenous and exogenous chemicals from void to void (Barr et al., 2005). Nonetheless, the reliability of non-persistent pesticide metabolites may be adequate when individuals are categorized into broad exposure groups due to their consistent individual time-activity patterns combined with constant microenvironmental concentrations that may lead to 'steady state' metabolite concentrations over longer time periods (Meeker et al., 2005). The ability of a single urine sample to predict metabolite concentrations over longer periods of interest and implications for detecting health outcomes in epidemiological studies need continued investigation. DAP metabolites are limited as biomarkers of exposure, as they do not retain the structure from which they were derived, and cannot be attributed to a specific original parent compound (Bravo et al., 2004). Over the years, the use of DAP metabolites as biomarkers of exposure for OP pesticides has been scrutinized. DAPs may also occur in the environment as a result of degradation of organophosphorus insecticides (Lu et al., 2005). The DAP metabolites can be present in urine after low level exposures to organophosphorus insecticides that do

not cause clinical symptoms or inhibition of cholinesterase activity (Davies and Peterson, 1997; Franklin et al., 1981). Moreover, the inability to distinguish exposures to parent compounds from exposures to pre-formed DAPs has been a concern (Krieger et al., 2012; Sudakin and Stone, 2011). The presence of DAPs in a person's urine may reflect exposure the parent OP pesticide or to the metabolites themselves. Therefore, there is the need to evaluate the effects and impact on spermatogenesis caused by the exposure to preformed degradation products versus exposure to parent compounds. Despite these limitations, biological measurements of urinary DAP metabolites are the most practical and widely used method to estimate the internal dose of most OP pesticides used worldwide (Angerer et al., 2007). These analyses continue to be extensively used in biomonitoring studies due to their relatively low costs and their utility for interpreting health outcomes of interest.

The men in this study population were recruited from a fertility clinic as opposed to the general population; however, there is no evidence that our participants would differ in their response to OP exposures. More than half of the men in our study population had normal semen motility, concentration and morphology, and should increase the generalizability of our results. In addition, extensive information on potential confounders was collected and adjusted for in the final models. We recognized that disomy frequencies in the current study are higher than other reported frequency distributions (Egozcue et al., 1997; Templado et al., 2005). However, those studies did not include men seeking fertility consultation. A validated semi-automated method for disomy frequency determination was used in this study. The semiautomated method allowed for reliable processing of a large number of samples (Perry et al., 2007a, 2011a).

The use of a semiautomated method is considered a strength in this study because it allowed for an objective counting of disomic sperm in a large number of samples.

Conclusion

This is the first epidemiologic study of this size to examine the relationship between environmental OP exposures and human sperm disomy outcomes. This study assessed environmental OP exposures and human sperm disomy outcomes by conducting multiple analyses adjusted for potential confounders. Our findings suggest that increased disomy rates were associated with specific DAP metabolites. Conversely, significant inverse associations were also observed between DMP exposure levels and most disomy outcomes. Total sum of DAP metabolites concealed individual associations observed for each individual metabolite. Dose-response relationships appeared nonmonotonic, with most of the increase in disomy rates occurring between the second and third exposure quartiles and without additional increases between the third and fourth exposure quartiles. These complex relationships present a challenge when documenting the associations between OP exposures and sperm chromosomal abnormalities. Future research needs to ascertain the mechanisms by which EDCs affect health reproductive outcomes. Detailed exposure assessments are also needed to further our understanding of sperm health effects associated with environmental exposures.

Table 3.1. Characteristics of MGH Men.

Table 3.1: Characteristics of study participants (n=159).	
Variable	**Mean ± SD**
Age	35 ± 5
BMI (kg/m^2)	28 ± 5
Race	**N (%)**
White	137 (86)
Black	5 (3)
Other	17 (11)
Hispanic ethnicity	
No	149 (94)
Yes	10 (6)
Semen Concentration	
<15 million/mL	16 (10)
Semen Morphology	
<4% normal	28 (18)
Semen Motility	
<32% motile	33 (21)
Abstinence time	
<=2 days	35 (22)
3-4 days	74 (47)
>=5 days	50 (31)
Smoking (n=2 missing)	
No	116 (74)
Current smoker	11 (7)
Former smoker	30 (19)

Table 3.2. Number of Sperm Nuclei Scored and Percent Disomy.

Table 3.2: Number of sperm nuclei scored and percent disomy (n=159).				
Variable	**Mean ± SD**	**Median**	**25th**	**75th**
Nuclei (n)	6,848 ± 4,815	5,503	2,939	9,976
%X18	38 ± 9	40	33	45
%Y18	37 ± 9	39	33	43
%XX18	0.4 ± 0.4	0.3	0.2	0.5
%YY18	0.4 ± 0.3	0.3	0.2	0.5
%XY18	1.1 ± 0.8	0.9	0.6	1.5
Total Disomy %	1.9 ± 1.3	1.6	1.1	2.5

Table 3.3. Distribution of Pesticide Metabolite Levels in Urine.

Table 3.3: Distribution of pesticide metabolite levels in urine (n=159).							
Metabolite [a] (ng/mL)	**Mean ± SD**	**Percentile**					**Range**
		25th	**50th**	**75th**	**90th**	**95th**	
Unadjusted							
DMP	11±25	<LOD	4	12	29	39	<LOD-271
DMTP	9±17	1	3	7	21	39	<LOD-148
DMDTP	1±2	<LOD	0.4	1	4	6	<LOD-10
DEP	4±8	0.1	1	4	9	15	<LOD-64
DETP	2±3	<LOD	1	1	4	9	<LOD-20
DEDTP	0.1±0.1	<LOD	<LOD	<LOD	0.3	0.4	<LOD-1
∑DAPs[b]	188±318	20	98	194	408	790	3-2,340
SG-adjusted							
DMP	11±24	<LOD	5	15	28	43	<LOD-259
DMTP	10±18	1	4	9	29	44	<LOD-122
DMDTP	1±3	<LOD	0.5	1	4	8	<LOD-15
DEP	4±7	0.2	1	4	9	14	<LOD-54
DETP	2±3	0.2	1	2	5	7	<LOD-17
DEDTP	0.1±0.1	<LOD	<LOD	0.1	0.3	0.4	<LOD-1
∑DAPs[b]	202±303	34	113	236	469	728	3-2,094
CR-adjusted (missing=2)							
DMP	8±16	<LOD	3	7	20	28	<LOD-148
DMTP	7±16	1	3	6	16	25	<LOD-122
DMDTP	1±2	<LOD	0.3	1	3	5	<LOD-19
DEP	2±5	0.2	1	2	7	11	<LOD-34
DETP	1±2	0.1	1	1	4	6	<LOD-16
DEDTP	0.1±0.1	<LOD	<LOD	0.1	0.2	0.5	<LOD-1
∑DAPs[b]	143±242	23	67	155	314	471	2-1,800

[a] LOD= 0.6 ng/mL (DMP), 0.2 ng/mL (DMTP), 0.2 ng/mL (DEP), and 0.1 ng/mL (DETP, DMDTP, DEDTP). Percent of samples above the LOD: DMP=57% (n = 91); DMTP=87% (n = 139); DMDTP=57% (n = 90); DEP=64% (n = 101); DETP=72% (n = 114); and DEDTP=10% (n = 16). [b] ∑DAPs urinary concentration units are nmol/mL.

Table 3.4. Aneuploidy Mean ± SD by Detection of Individual Urinary Organophosphate Metabolite Levels.

Table 3.4: Aneuploidy Mean ± SD by detection of individual urinary organophosphate metabolite levels of men seeking infertility evaluation (n=159).				
Metabolite[a]	**%XX18**	**%YY18**	**%XY18**	**%Total Disomy**
DMP				
Q1	0.49±0.48	0.39±0.37	1.27±0.98	2.15±1.53
Q2	0.45±0.59	0.31±0.20	1.00±0.61	1.76±1.09
Q3	0.36±0.29	0.39±0.26	0.92±0.74	1.67±1.06
Q4	0.38±0.32	0.31±0.17	1.13±0.68	1.81±0.95
DMTP				
Q1	0.36±0.29	0.37±0.21	1.15±0.68	1.88±0.89
Q2	0.52±0.59	0.40±0.43	1.21±1.18	2.12±1.86
Q3	0.42±0.40	0.37±0.27	1.01±0.60	1.81±1.01
Q4	0.41±0.41	0.31±0.18	1.13±0.69	1.85±1.01
DMDTP				
Q1	0.46±0.49	0.38±0.36	1.20±0.97	2.04±1.52
Q2	0.50±0.49	0.30±0.19	1.21±0.75	2.01±1.19
Q3	0.41±0.44	0.38±0.28	1.00±0.80	1.79±1.14
Q4	0.33±0.23	0.36±0.20	0.98±0.53	1.67±0.78
DEP				
Q1	0.37±0.26	0.35±0.25	1.11±0.69	1.83±0.97
Q2	0.58±0.64	0.47±0.45	1.35±1.26	2.40±1.98
Q3	0.36±0.43	0.32±0.23	0.86±0.56	1.53±0.93
Q4	0.48±0.44	0.30±0.15	1.18±0.65	1.95±1.00
DETP				
Q1	0.40±0.36	0.33±0.20	1.24±0.81	1.96±1.08
Q2	0.44±0.48	0.36±0.29	0.93±0.52	1.74±0.98
Q3	0.46±0.44	0.44±0.41	1.34±1.17	2.25±1.88
Q4	0.44±0.51	0.32±0.23	0.97±0.62	1.73±0.97
DEDTP				
G1	0.44±0.46	0.37±0.30	1.15±0.86	1.95±1.31
G2	0.43±0.33	0.28±0.16	0.93±0.51	1.64±0.85
∑DAPs				
T1	0.50±0.53	0.37±0.38	1.27±1.07	2.14±1.65
T2	0.46±0.48	0.40±0.29	1.08±0.74	1.94±1.17
T3	0.35±0.28	0.31±0.18	1.02±0.61	1.68±0.86

[a] DMP Exposure Quartiles: Q1=X≤LOD (n=68), Q2=0.60<X≤7.95 ng/mL (n=30), Q3=7.95<X≤13.39 ng/mL (n=30), Q4=X>13.39 ng/mL (n=31). DMTP Exposure Quartiles: Q1=X≤LOD (n=30), Q2=0.20<X≤2.21 ng/mL (n=43), Q3=2.21<X≤6.47 ng/mL (n=42), Q4=X>6.47 ng/mL (n=44). DMDTP Exposure Quartiles: Q1=X≤LOD (n=69), Q2=0.10<X≤0.73 ng/mL (n=30), Q3=0.73<X≤1.86 ng/mL (n=30), Q4=X>1.86 ng/mL (n=30). DEP Exposure Quartiles: Q1=X≤LOD (n=58), Q2=0.20<X≤1.46 ng/mL (n=34), Q3=1.46<X≤3.96 ng/mL (n=33), Q4=X>3.96 ng/mL (n=34). DETP Exposure Quartiles: Q1=X≤LOD (n=45), Q2=0.10<X≤0.62 ng/mL (n=39), Q3=0.62<X≤1.51 ng/mL (n=37), Q4=X>1.51 ng/mL (n=38). DEDTP Exposure Group: G1=X≤LOD (n=143); G2=X>0.10 ng/mL (n=16). ∑DAPs Exposure Tertiles: T1=2.76≤X≤35.00 nmol/mL (n=53), T2=35.00<X≤155.00 nmol/mL (n=52), T3=X>155.01 nmol/mL (n=54).

Table 3.5. Adjusted IRRs for each Disomy Outcome by Organophosphate Metabolite.

Table 3.5: Adjusted IRRs (95% CI) for XX18, YY18, XY18, and total sex-chromosome disomy by organophosphate metabolite of men seeking infertility evaluation (n=159).				
Metabolite[a]	**Adjusted IRRs**[b]			
	XX18	**YY18**	**XY18**	**Total Disomy**
DMP				
Q1	1.00	1.00	1.00	1.00
Q2	0.69 (0.63, 0.76)	1.00 (0.92, 1.09)	0.79 (0.74, 0.84)	0.81 (0.78, 0.84)
Q3	0.81 (0.74, 0.89)	0.89 (0.80, 0.99)	0.92 (0.87, 0.98)	0.89 (0.86, 0.93)
Q4	0.77 (0.70, 0.86)	1.07 (0.97, 1.18)	0.80 (0.75, 0.85)	0.84 (0.80, 0.88)
p-Value for Trend	<0.0001	0.2440	<0.0001	<0.0001
DMTP				
Q1	1.00	1.00	1.00	1.00
Q2	1.33 (1.18, 1.50)	1.09 (0.97, 1.23)	1.09 (1.02, 1.16)	1.13 (1.08, 1.19)
Q3	1.52 (1.36, 1.69)	1.03 (0.93, 1.15)	1.06 (1.00, 1.12)	1.14 (1.08, 1.20)
Q4	1.24 (1.10, 1.38)	1.21 (1.09, 1.35)	0.91 (0.86, 0.97)	1.03 (0.98, 1.08)
p-Value for Trend	0.1214	<0.0142	<0.4193	<0.0236
DMDTP				
Q1	1.00	1.00	1.00	1.00
Q2	1.22 (1.12, 1.34)	1.26 (1.15, 1.38)	0.98 (0.92, 1.03)	1.08 (1.04, 1.13)
Q3	1.24 (1.14, 1.36)	1.01 (0.92, 1.12)	1.28 (1.22, 1.35)	1.23 (1.18, 1.28)
Q4	1.01 (0.91, 1.13)	1.16 (1.05, 1.29)	1.10 (1.04, 1.17)	1.09 (1.04, 1.15)
p-Value for Trend	<0.0521	<0.0982	<0.0001	<0.0001
DEP				
Q1	1.00	1.00	1.00	1.00
Q2	1.23 (1.12, 1.35)	1.04 (0.94, 1.15)	1.07 (1.01, 1.13)	1.09 (1.05, 1.14)
Q3	1.13 (1.03, 1.24)	1.45 (1.33, 1.59)	1.09 (1.04, 1.15)	1.16 (1.11, 1.21)
Q4	0.92 (0.83, 1.02)	0.98 (0.88, 1.09)	0.84 (0.79, 0.89)	0.88 (0.84, 0.92)
p-Value for Trend	<0.0010	<0.5790	<0.7969	<0.1784
DETP				
Q1	1.00	1.00	1.00	1.00
Q2	1.07 (0.98, 1.18)	1.39 (1.26, 1.53)	0.99 (0.94, 1.05)	1.07 (1.02, 1.11)
Q3	1.13 (1.02, 1.24)	1.31 (1.18, 1.45)	0.89 (0.89, 0.95)	1.00 (0.96, 1.05)
Q4	0.83 (0.75, 0.92)	1.22 (1.09, 1.36)	0.73 (0.69, 0.78)	0.83 (0.79, 0.87)
p-Value for Trend	<0.0007	<0.0001	<0.0001	<0.0001
DEDTP[c]				
G1	1.00	1.00	1.00	1.00
G2	1.10 (0.99, 1.22)	0.85 (0.76, 0.96)	0.90 (0.84, 0.96)	0.93 (0.88, 0.98)
∑DAPs				
T1	1.00	1.00	1.00	1.00
T2	1.04 (0.96, 1.13)	1.36 (1.25, 1.48)	0.94 (0.90, 0.99)	1.03 (0.99, 1.07)
T3	0.85 (0.77, 0.93)	1.08 (0.98, 1.20)	0.86 (0.82, 0.91)	0.89 (0.86, 0.93)

p-Value for Trend	<0.0002	<0.1755	<0.0001	<0.0001

[a] DMP Exposure Quartiles: Q1=X≤LOD (n=68), Q2=0.60<X≤7.95 ng/mL (n=30), Q3=7.95<X≤13.39 ng/mL (n=30), Q4=X>13.39 ng/mL (n=31). DMTP Exposure Quartiles: Q1=X≤LOD (n=30), Q2=0.20<X≤2.21 ng/mL (n=43), Q3=2.21<X≤6.47 ng/mL (n=42), Q4=X>6.47 ng/mL (n=44). DMDTP Exposure Quartiles: Q1=X≤LOD (n=69), Q2=0.10<X≤0.73 ng/mL (n=30), Q3=0.73<X≤1.86 ng/mL (n=30), Q4=X>1.86 ng/mL (n=30). DEP Exposure Quartiles: Q1=X≤LOD (n=58), Q2=0.20<X≤1.46 ng/mL (n=34), Q3=1.46<X≤3.96 ng/mL (n=33), Q4=X>3.96 ng/mL (n=34). DETP Exposure Quartiles: Q1=X≤LOD (n=45), Q2=0.10<X≤0.62 ng/mL (n=39), Q3=0.62<X≤1.51 ng/mL (n=37), Q4=X>1.51 ng/mL (n=38). DEDTP Exposure Group: G1=X≤LOD (n=143); G2=X>0.10 ng/mL (n=16). ∑DAPs Exposure Tertiles: T1=2.76≤X≤35.00 nmol/mL (n=53), T2=35.00 <X≤155.00 nmol/mL (n=52), T3=X>155.01 nmol/mL (n=54).

[b] IRRs were adjusted for specific gravity, age, race, BMI, smoking, total concentration, motility, and morphology.

∑DAPs is the sum of all six individual metabolites. [c] No p-value for trend was calculated for adjusted disomy IRRs and DEDTP.

Chapter 4: Environmental Pesticide Exposure Interactions and Risk of Sperm Chromosomal Abnormalities

Introduction

The investigation of mechanistic interactions between environmental chemicals and reproductive health outcomes is a critical emerging research issue in environmental health and reproductive biology (Woodruff et al., 2008). Individual environmental toxicants have been shown to affect male reproduction, including infertility. Congenital abnormalities and unviable pregnancies have been linked to problems during spermatogenesis (Cheng et al., 2011), including poor sperm DNA integrity (Mruk and Cheng, 2011) and increased human sperm aneuploidy (Hassold and Hunt, 2001; Jacobs, 1992). Because sex chromosomes (X and Y) are particularly susceptible to aneuploidy, or errors in chromosome segregation during meiotic cell division, researchers have attempted to understand the paternal role in sex chromosome aneuploidy (Hassold and Hunt, 2001; Martin et al., 1991). Sex chromosome disomy is the most common type of aneuploidy, where two chromosomes are present in one germ cell. Disomic sperm do not prevent conception and can produce viable offspring with genetic conditions (Olson and Magenis, 1988; Chandley et al., 1991; Crow et al., 2000; Hassold and Hunt, 2001), reproductive disorders, behavioral and/or intellectual difficulties (Boyd et al., 2011; Martin, 2006). Despite advances in the study of chromosomal abnormalities, the exact causes of aneuploidy and/or the specific critical windows of chemical susceptibility that are associated with a heightened risk of aneuploidy remain unknown (Herrera et al., 2008; Axelsson et al., 2010; Ashton Acton, 2013).

Scientists have argued for years that real-life exposures to a specific environmental contaminant do not occur as single discrete events, but rather in combination to several toxicants at once (US EPA, 2001; WHO, 2009; Zeliger, 2011). Compounds in mixtures interact with each other and with biological systems (via additive, synergistic, potentiating, or antagonistic mechanisms), and they can greatly alter the toxicity of individual compounds even in low doses (Tsatsakis et al., 2009; Zeliger, 2011; Hernandez et al., 2013). Endocrine disrupting chemicals , such as pesticides, can modulate the endocrine system via primary or secondary mechanisms (McKinlay et al., 2008a; Mnif et al., 2011; Elersek and Filipic, 2011; Kang et al., 2004) and are present in the environment as complex mixtures. Humans are typically exposed to multiple EDCs at any one time via direct and/or indirect exposure pathways (Eil and Nisula, 1990; Tyler et al., 2000; Jørgensen et al., 2006).

Organophosphate (OP) and pyrethroid (PYR) insecticides account for a large share of all US insecticide use. OPs are most frequently used in agriculture, recreational and commercial areas, while PYRs are regularly used in homes and gardens, where an estimated 240 million applications of PYRs are made annually (US EPA, 2011). Mixtures of these pesticide are often found in the human food supply and aquatic environments. OPs and PYRs have been reviewed under the Food Quality Protection Act (FQPA) of 1996. FQPA directs the US Environmental Protection Agency (US EPA) to consider multiple pesticide exposures based on the determination that each pesticide family, which constitute more than one pesticide, share a common mechanism of toxicity. Although the federal government and independent organizations have expressed concern about the full domain of potential EDC impacts (US EPA, 2013; NAS, 2014), as yet,

regulatory efforts and current assessments do not consistently identify the nature of the interactive effects that may occur between the individual components of a pesticide mixture with different modes of action (Teuschler et al., 2004; Hernandez et al., 2013).

Urinary levels of OP (such as dialkyl phosphates or DAPs) and PYR (3-phenoxybenzoic acid or 3PBA) metabolites, have been measured in the general population (CDC, 2009). Adverse effects of OP and PYR exposures have been demonstrated for hormone functions and levels (Meeker et al., 2006; Lacasaña et al., 2010; Meeker et al., 2009), semen parameters and sperm DNA damage/fragmentation (Yucra et al., 2008; Recio-Vega et al., 2008; Perry et al., 2007b; Hossain et al., 2010; Meeker et al., 2004b; Ji et al., 2011, Lifeng et al., 2006, Xia et al., 2008; Meeker et al., 2008, Toshima et al., 2012), sperm chromatin structure alteration (Sanchez-Pena et al., 2004) and sex chromosome disomy in human sperm (Padungtod et al., 1999; Recio et al., 2001; Young et al., 2013; Radwan et al., 2015; Chapter 3 of Dissertation).

To my knowledge, there is very limited information about the reproductive health effects of environmental exposure to pesticide mixtures and their association with sperm chromosomal abnormalities. This study focuses on the potential interactions of OP and PYR pesticides and their association with altered frequency of sperm sex chromosome disomy among adult men. This exploratory analysis aimed to examine the combined effects of pesticide mixtures which may account for unexplained health effects and/or associations that each chemical would not produce independently.

Materials and Methods

Study Subjects

The participants in this study (n=159) were selected from a previous study of couples seeking infertility evaluation at Massachusetts General Hospital (MGH) Fertility Center. The parent study (n=341) was conducted between January 2000 and May 2003 and assessed the impact of environmental exposures on semen quality. Detailed description of the parent study has been described elsewhere (Hauser et al., 2003). Approximately 65% eligible subjects from the parent study aged 20-54 agreed to participate in the parent study; lack of participation was due to lack of time available during their clinic visit. Men receiving treatment for infertility and/or scheduled for post-vasectomy semen analysis were excluded from the parent study. Information on demographics, medical and fertility history, and lifestyle factors were obtained by a self-administered questionnaire. Data on potential confounding variables was also collected. Urine and semen samples were collected on the same day. Occupational exposure to pesticides or other agents was not reported by any participant (i.e., study population was selected without attention to specific occupational exposure). Because biological measurements (i.e., urine and semen) from the parent study (n=341) have been used for other analyses, eligibility for this study was based on samples availability in the biorepository (n=159 or 47%). An informed consent signed form was obtained from each participant. The parent study was approved by the Harvard School of Public Health, the Massachusetts General Hospital Human Subjects Committees, and by the Office of Human Research at the George Washington University.

Semen Analysis

Semen measurements have been previously described (Hauser et al., 2003). Semen samples were collected at the clinic via masturbation. Participants were asked to abstain from ejaculation at least for 48 hours prior to sample collection. Semen samples were liquefied at 37ºC for 20 minutes before analysis. Andrologists from the MGH Andrology Laboratory analyzed the samples; they were blinded to exposure status when the semen samples were analyzed. Volume, pH, color, and viscosity properties were determined for each semen sample. A computer-aided sperm analysis (CASA) using the Hamilton-Thorn Motility Analyzer (10HTM-IVO) was used to determine sperm count and percent motility. Two slides per sample were prepared for a morphological assessment. An oil immersion microscope lens with a 100x objective was used for this analysis (Nikon Company, Tokyo, Japan). Criteria reported by Kruger et al. (1988) was used to score sperm normal or abnormal.

Disomy Analysis

Semen samples were stored in -80°C without cryoprotectant until FISH analysis was performed. Disomy detection procedures are published elsewhere (McAuliffe et al., 2012). Laboratory technicians were blinded to exposure status. Fluorescence *in situ* hybridization (FISH) analysis was performed for chromosomes X, Y and 18 (autosomal control) to determine XX18, YY18, XY18 and total sex chromosome disomy in sperm nuclei. The primary outcome of interest was sex chromosome disomy, which is the most common form of sperm aneuploidy, capable of producing X or Y disomic sperm suitable for fertilization and viable offspring. Disomy in the sex chromosome is two times more frequent than disomy in the autosomes. For each FISH slide, a sequence of non-

overlapping field images were taken. A fluorescence microscope was utilized to take images subsequently scored for size and shape using custom MATLAB software and criteria as reported by Baumgartner et al. (1999). This semi-automated scoring method have been reported previously by Perry et al. (2011a).

Urinary OP and PYR Measurements

Urinary samples were analyzed according to previously published guidelines (Prapamontol et al., 2013). Human exposure to OP pesticides was estimated using DAP urinary metabolites (i.e., DMP, DMTP, DMDTP, DEP, DETP, DEDTP). Gas chromatography (GC) coupled with flame photometric detection (FPD) with isotopic dilution quantification was used to analyze all six DAP metabolites. Each metabolite was estimated by spiking pooled urine samples using a serial dilution of the lowest standard calibration solution to obtain a concentration providing a signal-to-noise ratio (S/N) of ≥3. The limits of detection (LOD) for each DAP metabolite were 0.6 ng/mL (DMP), 0.2 ng/mL (DMTP), 0.2 ng/mL (DEP), and 0.1 ng/mL (DETP, DMDTP, DEDTP). Transformed metabolite concentrations (i.e., each DAP metabolite divided by its molecular weight) were summed and multiplied by 1,000 to obtain a total DAPs (ΣDAP) concentration in units of nmol/mL. Furthermore, the urinary 3PBA metabolite level was used to estimate human exposure to PYR pesticides. Urine samples were analyzed using a small modification of the method of Baker et al., 2004. The samples were spiked with an isotopically labeled analogue to enable isotope dilution quantification. The target analyte was isolated using solid phase extraction. The extract was concentrated prior to analysis by high performance liquid chromatography-tandem mass spectrometry using an Agilent 6460 triple quadruple mass spectrometer (Santa Clara, CA) with Jetstream

electrospray ionization. Quality control and blank samples were analyzed jointly with unknown samples to ensure method stability and robustness. The LOD was 0.1 ng/mL with relative standard deviations of <15%.

Specific gravity and creatinine levels were measured in urine samples using a handheld refractometer (National Instrument Company, Inc., Baltimore, MD, USA) and kinetic colorimetric assay technology with a Hitachi 911 automated chemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA), respectively.

Statistical Analysis

Descriptive statistics were generated for demographic and semen parameter variables. Semen parameters were dichotomized using the World Health Organization reference values for sperm concentration (<15 million sperm/mL) and motility (<32% motile sperm), and the Tygerberg Strict Criteria for morphology (<4% normal morphology) (Kruger et al., 1988; World Health Organization 2010). For metabolite values below the LOD, an imputed value equal to one-half the LOD was used. Descriptive statistics for pesticide metabolite levels (μg/L) in urine were summarized. Creatinine and specific gravity adjusted summaries as well as volume-based (unadjusted) values were also calculated. Although creatinine is frequently used to adjust for urine dilution, specific gravity is considered to be more appropriate to adjust urinary metabolite concentrations (Barr et al., 2005).

Poisson regression (SAS GENMOD procedure) was used to model the association between each specific DAP and 3PBA volume-based urinary metabolite concentrations and the disomy measures (i.e., XX18, YY18, XY18, and total sex chromosome disomy)

due to the large number of sperm being scored and the relatively low frequency of disomy. The number of sperm scored and the number of disomic nuclei were summed separately for each subject; the individual subject was treated as the unit of analysis. The natural logarithm of the number of sperm counted was used as the offset variable to standardize across subjects.

Models were fitted using a disomy measure as the outcome variable (i.e., as a count of disomic cells for XX18, YY18, XY18, and total sex chromosome disomy) and the metabolites of interest as the independent variables. Age, body-mass index (BMI), motility, morphology, log of sperm concentration and specific gravity were included as a continuous covariates. Also, smoking (never, former, and current), race (white, black, or other) and urinary metabolites (quartiles for most metabolites) were included as covariates. A log transformation of sperm concentration was used in the models as the distribution of sperm concentration is generally non-normal and positively skewed (Berman et al., 1996). Incidence rate ratios (IRRs) and 95% confidence intervals were calculated for each model.

Interactions between each DAP metabolite and 3PBA in association with each disomy outcome were examined. If an interaction was significant, the model IRRs for a specific DAP metabolite were calculated within each quartile of 3PBA. Linear tests for trend for most DAP metabolites and ∑DAP were also performed by 3PBA strata to further investigate the interaction. If an interaction was not significant, the interaction term was removed from the model. Statistical analysis was performed using SAS version 9.3 (SAS Institute Inc, Cary, NC).

Results

Table 4.1 shows the demographic and semen parameter characteristics of the study subjects (n=159). The men had an average age of 35.3 years and a mean BMI of 28.0 kg/m^2. The majority of the men were white (86%) and non-Hispanic (94%). Most men (74%) had never smoked and 7% were current smokers. Of the 159 men, 10% (n = 16) had sperm concentrations <15 million/mL, 21% (n = 33) had <32% motile sperm, and 18% (n = 28) had <4% normally shaped sperm. A median of 6848 sperm nuclei were scored per subject (Table 4.2). The observed median percentages of XX18, YY18, XY18, and total disomy were 0.43, 0.36, 1.12 and 1.92, respectively. Table 4.3 summarizes the unadjusted urinary OP and PYR metabolites as well as the specific gravity and creatinine adjusted concentrations. The percent of samples above the LOD ranged from 57-89% for most metabolites.

Table 4.4 summarizes the significance (p-value) of the individual exposure variables (DAP metabolite and 3PBA) and the interaction term for each disomy measure. Statistically significant interactions were found between several DAP metabolites and 3PBA for XX18, YY18 and XY18 outcomes. A statistically significant interaction between all DAP metabolites and 3PBA was consistently observed for total disomy. The significance of all the interaction terms persisted when looking at continuous exposure terms.

Increased disomy rates were observed for XX18, YY18, XY18, and total sex chromosome disomy by levels of DMTP, DMDTP, DEP and DETP metabolites and 3PBA exposure (see Appendix C). The highest significant associations in the Appendix C tables were observed between a) XX18 and the second exposure quartile of DMDTP

(0.10-0.73 ng/mL) and third 3PBA exposure quartile (0.61-0.83 ng/mL) for an IRR = 3.35 (95% CI: 2.15, 5.20), and b) YY18 and the third exposure quartile of DETP (0.62-1.51 ng/mL) and second 3PBA exposure quartile (0.10-0.61 ng/mL) for an IRR = 4.13 (95% CI: 3.10, 5.49). Statistically significant inverse associations were also observed for all disomy types by levels of DMP and DEDTP metabolites and 3PBA exposure (see Appendix C). Increase in disomy rates occurred mainly between the second and third exposure quartiles and without substantial additional increases between the third and fourth exposure quartile, producing nonmonotonic dose response curves.

To further investigate interactions between OP and PYR metabolites, graphs of the adjusted IRRs and 95% confidence intervals were generated and examined for total sex chromosome disomy by quartiles of DAP metabolite (DMP, DMTP, DMDTP, DEP, DETP) and 3PBA quartiles (Figures 4.1-4.5; see also Appendix D for additional graphs). Nonmonotonic patterns were observed across most quartiles of 3PBA with increasing individual DAP exposure.

Inverse and positive parameter estimates (β) were determined to be significant using a test for trend across DAP metabolites by most 3PBA quartiles. Both significant U-shaped (Figure 4.3) and inverted-U shaped (Figure 4.4) relationships were observed across 3PBA quartiles with increasing DAP metabolites. Figure 4.1 shows a significant decreasing trend in 3PBA with increasing DMP exposure. Yet, there was an increasing trend in the upper quartile of 3PBA with increasing DMP exposure. In Figure 4.2, an increasing trend was observed in the second quartile of 3PBA, whereas the fourth quartile of 3PBA showed a decreasing trend with increasing DMTP exposure. Increased risks were consistently detected for total disomy in the second DMDTP exposure quartile

across all 3PBA quartiles; adjusted IRRs ranged from 1.36 (95%CI: 1.11, 1.67) to 1.96 (95%CI: 1.59, 2.40) (Figure 4.3). Similar patterns were observed for DEP exposure in all 3PBA exposure quartiles (Figure 4.4). Figure 4.5 shows increased risks for total disomy consistently detected in the third DETP quartile across all 3PBA quartiles; adjusted IRRs ranged from 1.28 (95%CI: 1.08, 1.52) to 2.31 (95%CI: 2.02, 2.64). Figure 4.6 shows adjusted IRRs and 95% confidence intervals for total disomy by tertiles of ∑DAP by 3PBA tertiles. Even though some associations were detected by the test for trend (p values=0.0001) in the first and third tertiles of 3PBA with increasing ∑DAPs exposure, small or no visible changes were observed.

Discussion

To our knowledge, this the first epidemiological study to evaluate the relationship between pesticide interactions and human sperm disomy outcomes. Significant increased rates were observed for XX18, YY18, XY18, and total sex chromosome disomy by levels of DMTP, DMDTP, DEP and DETP metabolites at various 3PBA levels after adjusting for potential confounders. Conversely, inverse associations were observed for all disomy types by levels of DMP and DEDTP metabolites and various 3PBA levels in adjusted models. In this study, increased IRRs, higher than the rates previously reported for each individual chemical class, were observed when assessing disomy outcomes and OP/ PYR mixtures. Adjusted IRRs for XX18, YY18, XY18, and total sex chromosome disomy by ∑DAPs and 3PBA exposures showed mainly inverse associations when compared to the reference group. These results confirmed our previous observation that aggregating all six DAP metabolites into a composite variable of ∑DAPs conceals their separate and distinct associations with disomy outcomes.

Our results showed complex nonmonotonic relationships, characterized by dose-response curves changing direction within the range of exposure categories. The highest disomy rates were seen either: 1) at the intermediate exposure level (i.e., quartiles 2 or 3) with low or no association observed at low and high exposures (inverse U-shaped relationship); or 2) with the highest rates observed at low and high exposures (U-shaped relationship). Men in this study were not significantly different from each other in terms of their demographic characteristics or semen parameters, and therefore, it is unlikely these nonmonotonic effects can be explained by differences across exposure groups.

Unadjusted 95th percentile DAP urinary concentrations were slightly higher (0.39-38.71 ng/mL) in this study when compared to the unadjusted urinary concentrations of males surveyed in the US general population, which ranged from 0.78-31.10 ng/mL for 2001-2002 and from <LOD-36.10 ng/mL for 2007-2008 (CDC, 2009 - Updated Tables 2015). The unadjusted 3PBA 95th percentile urinary concentrations were lower in this study (2.27 ng/mL) than the concentrations reported for 2001-2002 (3.23 ng/mL) and 2009-2010 (6.50 ng/mL).

Several human studies have reported individual exposures of OP or PYR pesticides associated with decreased semen quality (Meeker et al., 2004a), sperm volume and decreased sperm count (Yucra et al., 2008; Recio-Vega et al., 2008; Lifeng et al., 2006), semen quality and DNA damage (Xia et al., 2008; Meeker et al., 2008; Ji et al., 2011), sperm chromatin alteration (Sanchéz-Peña et al., 2004), increased luteinizing hormone and decreased testosterone (Padungtod et al., 1998), higher abnormal morphology and decreased sperm motility (Tan et al., 2002; Kamijima et al., 2004; Hossain et al., 2010), and sperm aneuploidy (Padungtod et al., 1999; Recio et al., 2001, Xia et al., 2004; Young

et al., 2013; Radwan et al., 2015). Less is known about the effects of OP/PYR pesticide mixtures and their association with human sperm parameters. Perry et al. (2007b) conducted a pilot biomonitoring study to examine the relationship between environmental OP/PYR exposure mixtures and sperm concentration among Chinese men living in rural areas. Results showed a high prevalence of exposure to OP/PYR pesticides and suggested that the higher exposure group had lower sperm concentration.

An animal study evaluated the reproductive effects in adult rats orally exposed to a mixture of five pesticides (Perobelli et al., 2010). Sperm motility was significantly decreased in animals exposed to the pesticide mixture. Their results suggested that the reproductive effects not seen with individual compounds may occur in the presence of several pesticides (Perobelli et al., 2010).

OP toxicity involves the inhibition of the enzyme acetylcholinesterase (AChE) leading to neurotoxicity in the central and peripheral nervous system (US EPA, 1999), while PYRs act via the activation and inactivation of voltage-gated sodium channels (VGSCs), causing neuronal excitability (Barr et al., 2010; Mandhane and Chopde, 1997; Nasuti et al., 2003). Because the use of PYRs in the US, particularly in residential settings, has increased dramatically over the past decade, it is important to consider their effects when combined with other pesticides that have higher mammalian toxicity such as the OPs (Barr et al., 2010; Horton et al., 2011; U.S. EPA, 2013; Williams et al., 2008). Pesticide mixtures can result in amplified health effects due to an increased toxicity of each compound (Hayes et al., 1991). Exposure to CYP450-activated OP insecticides or their toxic metabolites (such as chlorpyrifos oxon) can potentially enhance PYR toxicity by inhibiting carboxylesterases (enzymes used by the body to detoxify PYRs) so greater

toxicity is often observed (Ray et al., 2000). With metabolic detoxification inhibited and sometimes irreversible even at low concentrations, the toxic potency of PYR pesticides in the presence of OPs may be increased due to greater tissue concentration and lower excretion rate (Wielgomas and Krechniak, 2007). Both classes of insecticides are often used simultaneously in the US and are formulated in combined products on the global market, presenting evidence for the risk of unintended synergistic or potentiation effects (He et al., 2002). While there is a large body of literature on individual OP and PYR toxicology, all biological effects and synergistic mechanisms are not well understood (US EPA, 2009) and there is limited information about OP/PYR mixture effects on organs such as the testis.

Epidemiological studies among non-occupationally exposed adult males have found inverse and increased associations between individual OP exposure and hormones functions/levels (Recio et al., 2005; Meeker et al., 2006; Yucra et al., 2008; Lacasaña et al., 2010; Blanco-Muñoz et al., 2010). In addition, environmental exposure to PYR insecticides has been associated with changes in thyroid and sex hormone levels in men (Han et al., 2008; Meeker et al., 2009). Studies have also demonstrated PYRs ability to bind and disrupt androgen receptors in human reporter gene assays (Sun et al., 2007; Du et al., 2010). Molecular or physiological modes of action of environmental contaminants at low doses are likely to impact the endocrine system; however, their co-occurrence is difficult to discern (Welshons et al., 2003).

The findings here are consistent with potential low dose effect mechanisms responsible for affecting hormone expression and influencing human disorders (Vandenberg et al., 2012). Our findings showed complex nonmonotonic dynamics between OP/PYR

exposure mixtures and disomy. Even though the effects of low doses cannot be predicted by the effects observed at high doses and sometimes low environmental exposures cannot be identified (Vandenberg et al., 2012), this study was able to detect significant associations between pesticide mixtures and disomy. It is still uncertain how OP/PYR exposure mixtures might be protective for sex chromosome disomy when specific DAP metabolites were modeled within each quartile of 3PBA. Our results suggest that pesticide toxicity estimates may underestimate the risks associated with reproductive outcomes because they do not include the possibility of co-occurring synergistic chemical interactions.

Because chemical mixtures are multidimensional, there is not a standard methodology to investigate the effects of "real-life" exposures to mixtures of compounds with different modes of action (Teuschler et al., 2004, 2007; Perobelli et al., 2010). This highlights the difficulties with treating environmental exposures observed in epidemiological studies as single entities (EEA-JRC, 2013). Environmental exposures are highly correlated, introducing methodological issues. Several methodological advances have been made to account for issues such as collinearity, high dimensionality, and synergistic, potentiation or inhibitory effects. Advanced methods have been designed to identify subsets of mixtures (Gennings et al., 2010), assess mixtures that are also affected by a limit of detection (Herring, 2010), accommodate joint analysis of high-dimensional biomarker data (Zhang et al., 2012), and to model interactions (Charles et al., 2002; Moser et al., 2005, 2006; Yeatts et al., 2010; VanderWeele and Tchetgen, 2014). However, some of these methods are relatively new and have been primarily demonstrated using linear and logistic regression. Additional evidence of these type of interactions are needed in other

non-logistic models, such as Poisson regression which is best suited to the nature of the disomy rate data examined here.

This exploratory analysis was limited by the number of men available in each quartile when stratifying by exposure category. Commonly used trend tests available to evaluate monotonic dose-response relationships present a disadvantage when evaluating nonmonotonic responses. Those tests may not fully describe the nonmonotonicity patterns often observed for EDCs.

DAPs and 3PBA are non-specific urinary metabolites of OPs and PYRs insecticides. These metabolites do not retain the structure from which they were derived, and cannot be attributed to a specific original parent compound (Bravo et al., 2004). Although the use of biological measurements has been criticized due to the inability to distinguish parent compound exposures from exposures to other pre-formed breakdown products (Lu et al., 2005; Sudakin et al., 2006; Starr et al., 2008; Sudakin and Stone, 2011; Krieger et al., 2012), these urinary biomarkers are still the most widely used method to estimate the internal dose of a wide range of pesticides (Angerer et al., 2007) due to their relatively low costs and their utility for interpreting health outcomes of interest. Even though the existing multi-analyte methods are highly sensitive and able to measure low levels of exposure biomarkers in urine (Needham et al., 2007), detailed time-specific information on windows of exposure vulnerability are still needed to improve exposure assessment. Although our study population was recruited from a fertility clinic, their response to pesticide exposures is not expected to differ from men in the general population. About 50% of our study participants had normal semen parameters, including men with a range of semen parameter characteristics. Our data suggest that the observed associations do

not differ according to whether or not men had previous infertility exams. All these factors increase the generalizability of our results. Samples for 159 men (47%) were available from men enrolled in the parent study (n=341), which is considered high for a men's health study. It is important to acknowledge that the limitations of this cross-sectional study are ameliorated given the temporality of the exposure and outcome. Extensive information was collected about potential confounders. Semen measurements reflect recent spermatogenic cycles (75-90 days), and DAP and PYR urinary metabolites reflect recent exposure to pesticides. A high quality semi-automated method was used to determine disomy frequency in this study, allowing for a reliable and an objective counting of disomic sperm in a large number of samples (Perry et al., 2007a, 2011a). In addition, the technicians who performed semen and disomy analyses were blinded to exposure status, increasing the integrity of the results. Disomy frequencies in this study are higher than some of the frequency distributions previously reported (Egozcue et al., 1997; Templado et al., 2005, 2011).

Conclusion

This is the first epidemiologic study to explore the relationship between pesticide mixtures and human sperm disomy outcomes. This exploratory analysis intended to examine the interactions between OP and PYR pesticide mixtures and human sperm disomy outcomes by conducting multiple statistical analyses adjusted for potential confounders. Increased IRRs, higher than the rates previously detected for each individual chemical class, were observed when evaluating the associations between sex chromosome disomy and pesticide mixtures. Our findings suggest that compounds with different modes of action could have synergistic and/or potentiation effects and modify

the independent toxicity and health effects of the individual compounds. Nonmonotonic associations between pesticide interactions and health reproductive outcomes are challenging to interpret. Our results show that chemical interactions could potentially increase risks of total sperm disomy. Our outcome of interest, disomy, is best modeled assuming a Poisson distribution. Methods to disentangle the effects of mixtures needed further development to optimally model outcomes that do not routinely fit linear or logistic distributions. Specific attention needs to be given to the methodologies available to assess the combined effects of simultaneous low-level pesticide exposures (with different modes of action and using non-logistic models) to enhance our ability to predict reproductive impacts of environmental contaminants. Because this is the first analysis conducted with this combination of chemicals for this health outcome, replication and extension of our findings is needed before the association between pesticide mixtures and aneuploidy can be fully elucidated.

Table 4.1. Characteristics of MGH Men.

Table 4.1: Characteristics of MGH men (n=159).	
Variable	**Mean ± SD**
Age	35.3 ± 5.4
BMI (kg/m^2)	28.0 ± 5.0
Race	**N (%)**
White	137 (86.2)
Black	5 (3.1)
Other	17 (10.7)
Hispanic ethnicity	
No	149 (93.7)
Yes	10 (6.3)
Semen Concentration	
<15 million/mL	16 (10.1)
Semen Morphology	
<4% normal	28 (17.61)
Semen Motility	
<32% motile	33 (20.8)
Abstinence time	
<=2 days	35 (22.0)
3-4 days	74 (46.5)
>=5 days	50 (31.5)
Smoking (n=2 missing)	
No	116 (73.9)
Current smoker	11 (7.0)
Former smoker	30 (19.1)

Table 4.2. Number of Sperm Nuclei Scored and Percent Disomy.

Table 4.2: Number of sperm nuclei scored and percent disomy of men seeking infertility evaluation (n=159).				
Variable	**Mean ± SD**	**Median**	**25th**	**75th**
Nuclei (n)	6848 ± 4815	5503	2939	9976
%X18	37.63 ± 9.07	39.75	33.29	44.76
%Y18	36.51 ± 8.81	39.15	32.78	43.33
% XX18	0.43 ± 0.44	0.28	0.17	0.52
% YY18	0.36 ± 0.29	0.31	0.19	0.46
% XY18	1.12 ± 0.83	0.90	0.57	1.53
Total Disomy %	1.92 ± 1.27	1.55	1.12	2.47

Table 4.3. Distribution of Individual Pesticide Metabolite Levels in Urine.

Table 4.3: Distribution of individual pesticide metabolite levels in urine of men seeking infertility evaluation (n=159).							
Metabolite [a] (ng/mL)	**Mean ± SD**	**Percentile**					**Range**
		25th	**50th**	**75th**	**90th**	**95th**	
Unadjusted							
DMP	10.77±25.30	0.30	4.34	12.48	29.42	38.60	0.30-270.75
DMTP	8.62±17.42	0.68	3.13	7.28	21.11	38.71	0.10-148.12
DMDTP	1.18±2.03	0.05	0.43	1.16	3.51	5.96	0.05-10.32
DEP	3.68±8.24	0.10	1.00	3.65	8.87	14.82	0.10-63.56
DETP	1.70±3.48	0.05	0.60	1.46	3.83	9.27	0.05-20.08
DEDTP	0.09±0.12	0.05	0.05	0.05	0.26	0.39	0.05-0.66
3PBA	0.89±1.51	0.51	0.62	0.87	1.57	2.27	0.05-15.18
SG-adjusted							
DMP	11.35±24.04	0.51	5.17	15.39	27.51	43.11	0.20-258.98
DMTP	9.76±18.07	0.85	3.73	9.13	28.62	44.19	0.05-121.76
DMDTP	1.41±2.52	0.08	0.46	1.30	4.46	8.36	0.03-15.13
DEP	3.72±7.48	0.22	1.33	4.14	9.33	13.70	0.05-53.78
DETP	1.77±2.92	0.16	0.76	1.71	4.51	7.04	0.03-16.98
DEDTP	0.11±0.13	0.05	0.06	0.11	0.29	0.43	0.03-0.72
3PBA	1.15±2.64	0.44	0.69	1.17	1.99	2.55	0.04-27.83
CR-adjusted (missing=2)							
DMP	7.87±16.26	0.47	2.57	7.24	19.80	27.86	0.12-147.95
DMTP	7.22±16.21	0.61	2.59	6.07	16.14	24.91	0.02-121.93
DMDTP	1.02±2.14	0.07	0.29	0.96	2.64	5.32	0.01-18.89
DEP	2.47±4.71	0.19	0.78	2.30	7.07	11.22	0.03-33.77
DETP	1.29±2.20	0.12	0.51	1.40	3.80	5.87	0.01-15.60
DEDTP	0.08±0.09	0.03	0.05	0.10	0.19	0.48	0.01-0.56
3PBA	1.08±3.92	0.28	0.47	1.01	1.72	2.24	0.02-46.56

[a] LOD= 0.6 ng/mL (DMP), 0.2 ng/mL (DMTP), 0.2 ng/mL (DEP), and 0.1 ng/mL (DETP, DMDTP, DEDTP, 3PBA). Percent of samples above the LOD: DMP=57% (n = 91); DMTP=87% (n = 139); DMDTP=57% (n = 90); DEP=64% (n = 101); DETP=72% (n = 114); and DEDTP=10% (n = 16); 3PBA=79% (n = 126).

Table 4.4. Significance of the Interactions between DAP Metabolites and 3PBA from Adjusted Models for each Disomy Outcome by OP Metabolite.

Table 4.4: Significance (p-value) of the interactions between DAP metabolites and 3PBA from adjusted models for XX18, YY18, XY18, and total sex chromosome disomy by OP metabolite of men seeking infertility evaluation.

Metabolite	XX18	YY18	XY18	Total Disomy
DMP				
DMP	0.8401	0.9348	0.4704	0.4310
3PBA	0.1741	0.0007	<.0001	<.0001
DMP*3PBA	0.0047	0.4183	0.0385	0.0015
DMTP				
DMTP	0.0083	0.6898	<.0001	<.0001
3PBA	0.1279	0.5586	0.0369	0.0176
DMTP*3PBA	0.1323	0.0737	<.0001	<.0001
DMDTP				
DMDTP	<.0001	<.0001	0.0007	<.0001
3PBA	0.0310	<.0001	<.0001	<.0001
DMDTP*3PBA	0.0085	0.0006	0.4837	0.0022
DEP				
DEP	<.0001	0.0010	<.0001	<.0001
3PBA	0.6838	<.0001	<.0001	0.3034
DEP*3PBA	<.0001	<.0001	<.0001	<.0001
DETP				
DETP	<.0001	<.0001	0.5650	<.0001
3PBA	0.0011	<.0001	<.0001	0.3413
DETP*3PBA	<.0001	<.0001	<.0001	<.0001
DEDTP				
DEDTP	<.0001	0.4437	0.0021	<.0001
3PBA	0.1125	<.0001	<.0001	<.0001
DEDTP*3PBA	<.0001	0.0073	<.0001	<.0001
∑DAPs				
DAPs	0.0836	0.6384	0.0588	0.0718
3PBA	0.0137	0.6514	0.1770	0.0780
DAPs*3PBA	<.0001	0.1451	<.0001	<.0001

- Model was adjusted for specific gravity, age, race, BMI, smoking, total concentration, motility, and morphology.
- ∑DAPs is the sum of all six individual metabolites.

Figure 4.1. Adjusted IRRs for Total Disomy by Quartiles of DMP by 3PBA Quartiles.

Adjusted IRRs (95% CI) for Total Disomy by Quartiles of DMP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

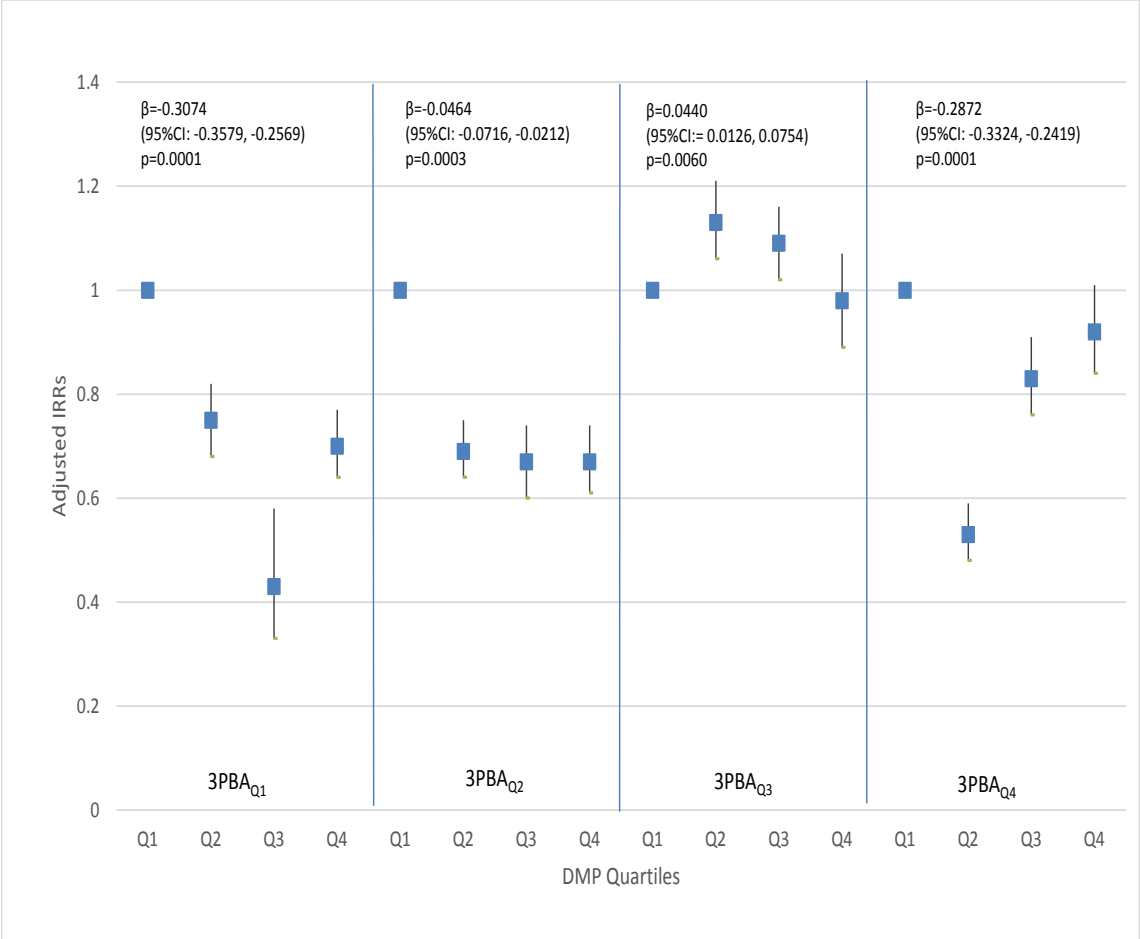


Figure 4.2. Adjusted IRRs for Total Disomy by Quartiles of DMTP by 3PBA Quartiles.

Adjusted IRRs (95% CI) for Total Disomy by Quartiles of DMTP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

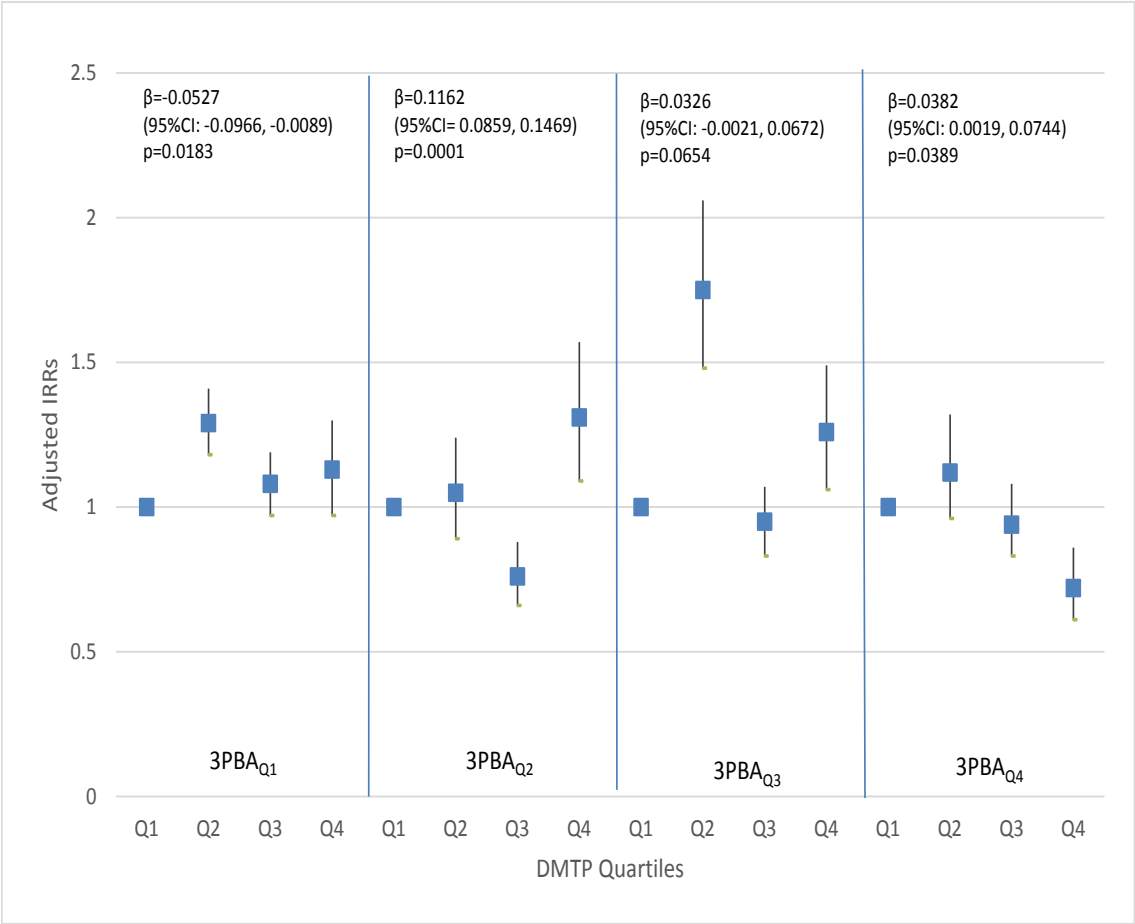


Figure 4.3. Adjusted IRRs for Total Disomy by Quartiles of DMDTP by 3PBA Quartiles.

Adjusted IRRs (95% CI) for Total Disomy by Quartiles of DMDTP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

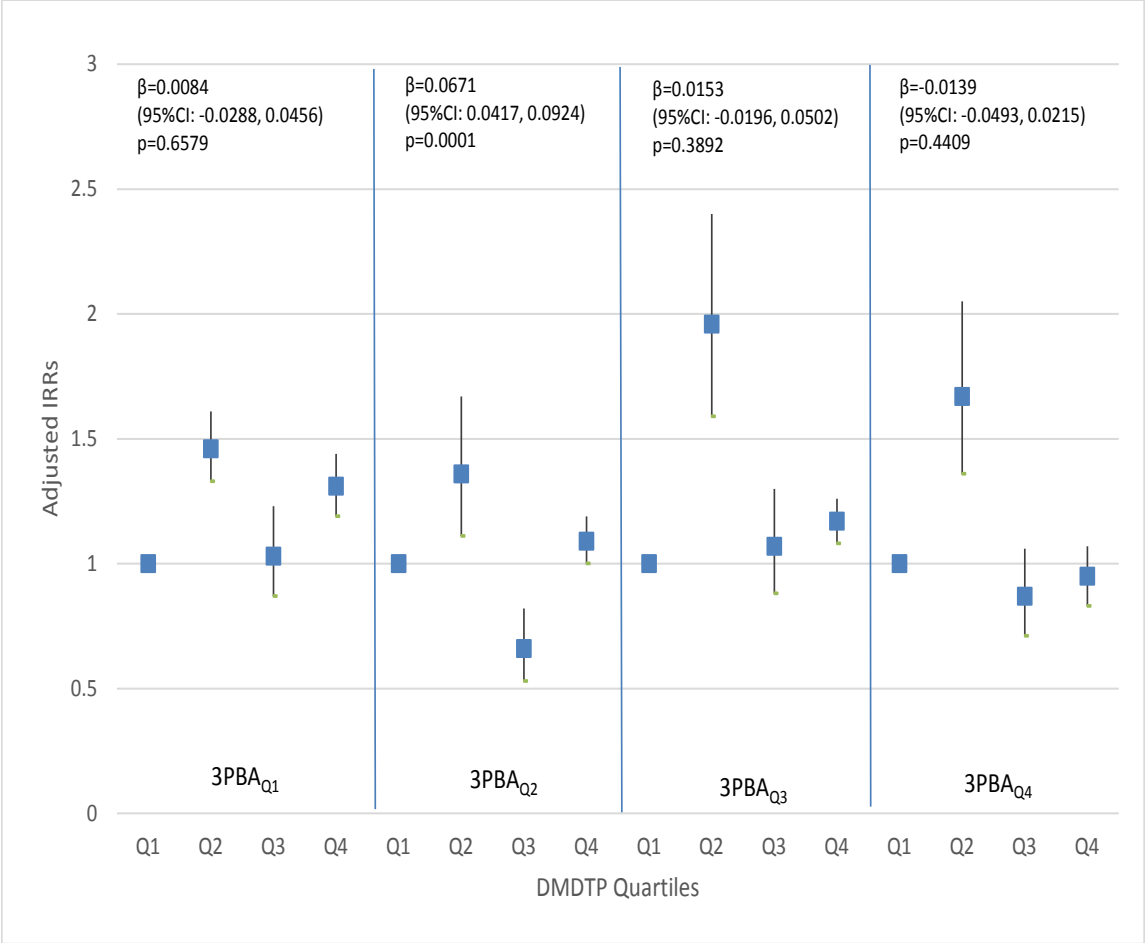


Figure 4.4. Adjusted IRRs for Total Disomy by Quartiles of DEP by 3PBA Quartiles.

Adjusted IRRs (95% CI) for Total Disomy by Quartiles of DEP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

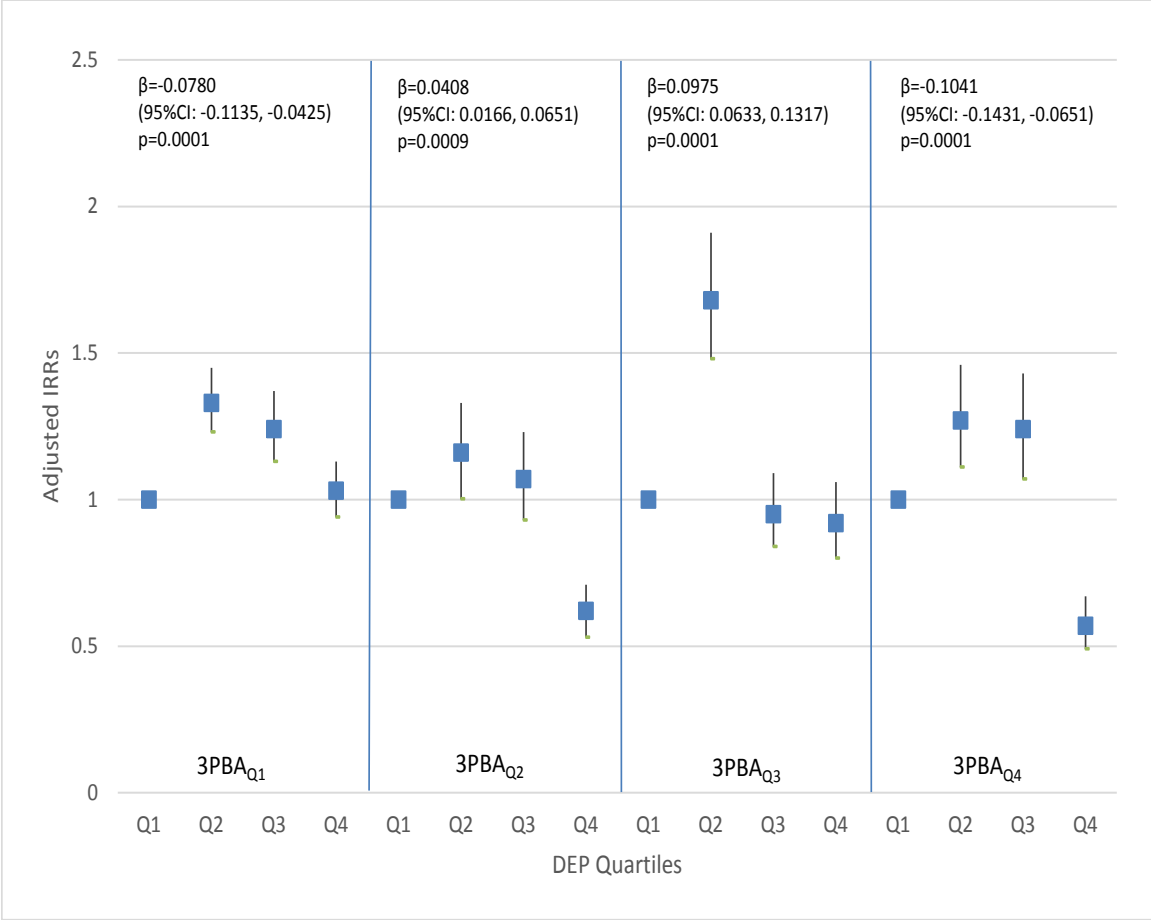


Figure 4.5. Adjusted IRRs for Total Disomy by Quartiles of DETP by 3PBA Quartiles.

Adjusted IRRs (95% CI) for Total Disomy by Quartiles of DETP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

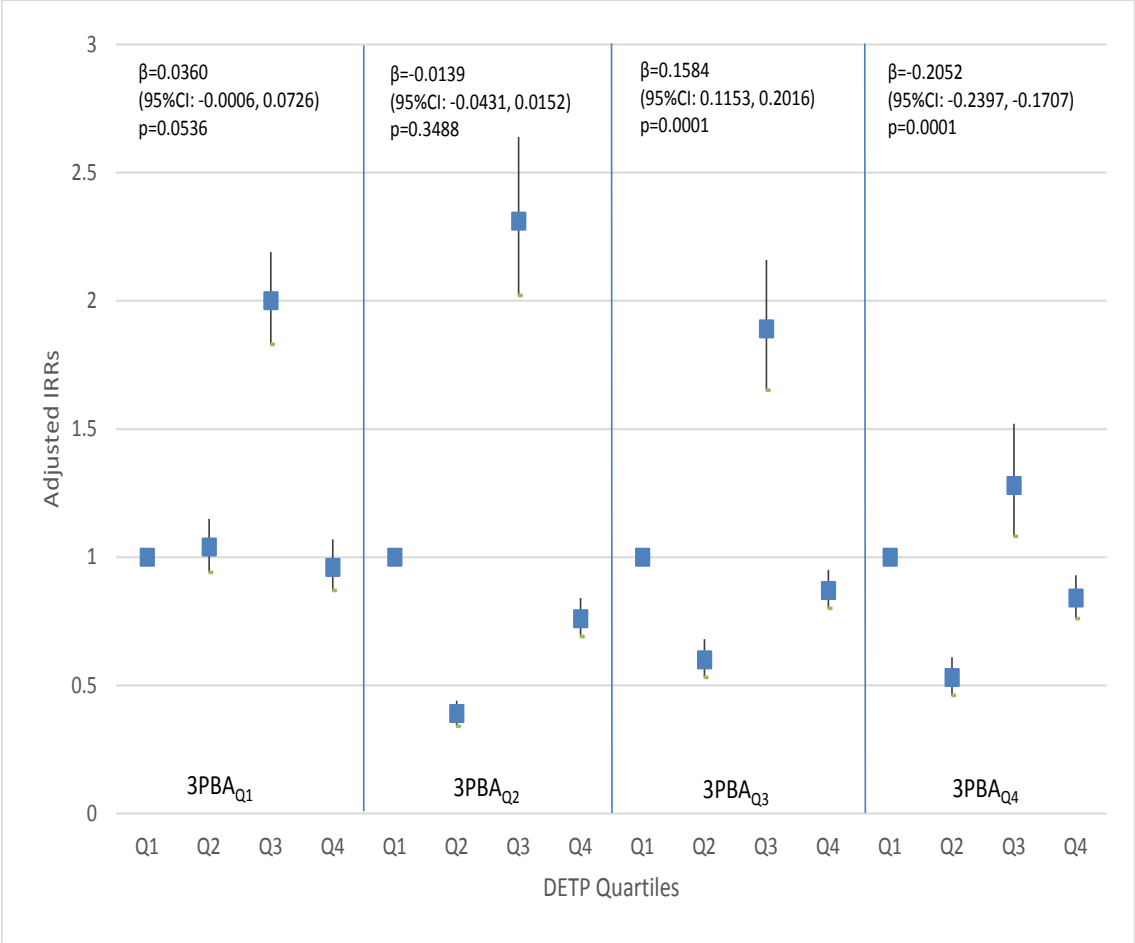
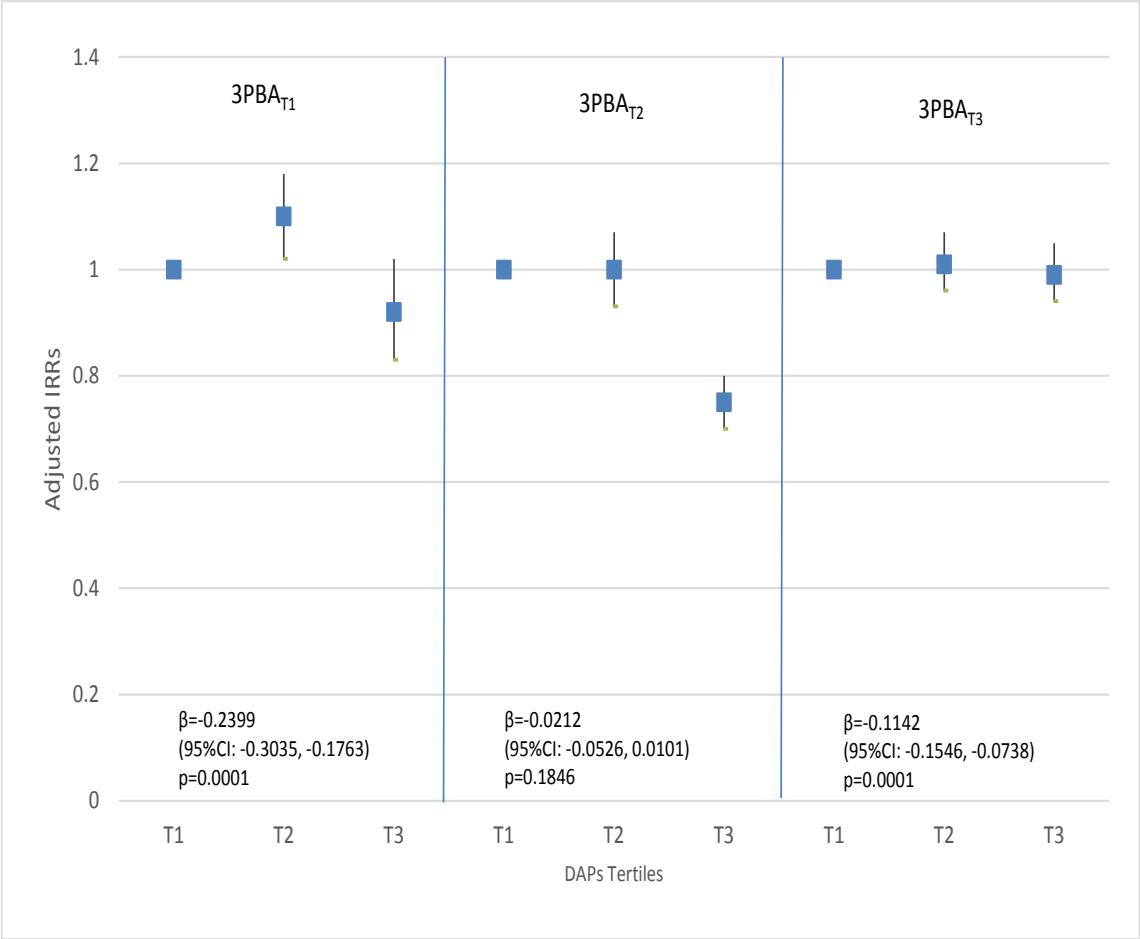


Figure 4.6. Adjusted IRRs for Total Disomy by Tertiles of DAPs by 3PBA Tertiles.

Adjusted IRRs (95% CI) for Total Disomy by Tertiles of DAPs (Exposure 1 changing) by 3PBA Tertiles (Exposure 2 constant).



Chapter 5: Conclusions

This dissertation research project examined environmental risk factors contributing to altered frequency of sperm sex chromosome abnormalities among adult men; evaluated the relationship between contemporary pesticide exposures and sperm aneuploidy (XX18, YY18, XY18 and total sex chromosome disomy); explored the health effects of pesticide mixtures, and documented potential interactions. This dissertation research project involved three separate investigations.

First, we investigated environmental exposure to pyrethroids (PYR) pesticides and their association with altered frequency of sperm sex chromosome disomy among a large sample of adult men. The following conclusions can be drawn from this specific study:

a) It is uncertain how increased exposure to endocrine disrupting chemicals (EDCs), such as PYR pesticides, might be protective for sperm chromosome disomy.
b) There are multiple mechanisms by which EDCs can modulate endocrine systems and potentially cause adverse reproductive effects.
c) Questions remain as to how environmental exposures to PYR pesticides affect sperm chromosomal abnormalities.

The second investigation examined environmental exposure to organophosphate (OP) pesticides and their association with the frequency of sperm sex chromosome disomy. This is the first epidemiologic study of this size to examine the relationship between environmental OP exposures and human sperm disomy outcomes. Based on the results the following conclusions can be made:

a) It is uncertain how increased exposure to OP pesticides might be protective for sperm sex chromosome disomy.
b) Total sum of OP metabolites concealed individual associations observed for each individual metabolite.
c) Nonmonotonic dose-response relationships present a challenge when documenting the associations between OP exposures and sperm chromosomal abnormalities.
d) Detailed exposure assessments are required to understand the health effects associated with sperm disomy and environmental exposures.
e) Future research is needed to ascertain the secondary mechanisms by which EDCs affect health reproductive outcomes.

Finally, we investigated environmental exposure to pesticide mixtures (PYR and OP) and their association with altered frequency of sperm sex chromosome disomy among adult men. This was the first epidemiologic study to explore the relationship between pesticide OP/PYR mixtures and human sperm disomy outcomes. This analysis suggests the following:

a) Pesticide mixtures and/or associations may account for some unexplained human health effects that each chemical class would not produce individually.
b) Compounds with different modes of action could have synergistic and/or potentiation effects and modify the independent toxicity and health effects of the individual compounds.

c) Chemical interactions could potentially increase the risk of sex chromosome disomy by enhancing the associations between environmental exposures and the frequency of sperm chromosomal abnormalities among men.
d) Due to their potential implications for human reproductive outcomes, it is important to assess interaction effects between pesticides commonly used in the US.
e) Specific attention needs to be given to the statistical methodologies available to assess nonmonotonic associations, especially when assessing combined effects of simultaneous low level pesticide exposures with different modes of action.
f) The statistical methodology used in this analysis is confined by the nature of the outcome. Further modeling work is needed to investigate chemical interactions using non-logistic models.

Because this is the first time an analysis was conducted with this set of chemicals for this health outcome, replication and extension of our findings in other epidemiological studies with different populations is needed before the association between pesticide mixtures and aneuploidy can be fully elucidated.

Overall these results demonstrate the impacts of environmental exposures on men's reproductive health. This original research is both relevant and contributes novel findings to the existing scientific literature. The recommendations provided herein will strengthen future investigations about the associations between environmental exposures and sperm sex chromosome disomy. Furthermore, because human exposure to environmental contaminants is not limited to an individual compound or class, it is and will continue to be challenging to predict the toxicity of mixtures based on our

knowledge about the toxicity and modes of action for single contaminants. These results will enlighten future epidemiological studies related to EDC mechanisms and environmental nonmonotonic dose-response relationships. We believe that these findings highlight the important role of environmental exposures in reproductive health outcomes.

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Appendix A – Organophosphate Pesticides and their potential Dialkyl Phosphate (DAP) Urinary Metabolites.

Table A.1: Organophosphate pesticides and their potential Dialkyl Phosphate (DAP) urinary metabolite (modified from Bravo et al., 2002).			
Dimethyl alkylphosphates (DMAPs)			
Parent Pesticide	**DMP**	**DMTP**	**DMDTP**
Azinphos-methyl	X	X	X
Chlorpyrifos-methyl	X	X	
Dichlorvos (DDVP)	X		
Dicrotophos	X		
Dimethoate	X	X	X
Fenitrothion	X	X	
Fenthion	X	X	
Isazaphos-methy	X	X	
Malathion	X	X	X
Methidathion	X	X	X
Methyl parathion	X	X	
Naled	X		
Oxydemeton-methyl	X	X	
Phosmet	X	X	X
Pirimiphos-methyl	X	X	
Temephos	X	X	
Tetrachlorviphos	X		
Trichlorfon	X		
Diethyl alkylphosphates (DEAPs)			
Chlorethoxyphos	X	X	
Chlorpyrifos	X	X	
Coumaphos	X	X	
Diazinon	X	X	
Disulfoton	X	X	X
Ethion	X	X	X
Parathion	X	X	
Phorate	X	X	X
Sulfotepp	X	X	
Terbufos	X	X	X

Appendix B – Adjusted IRRs for each Disomy Outcome by ∑DMAPs and ∑DEAPs Metabolites.

Table B.1: Adjusted IRRs (95% CI) for XX18, YY18, XY18, and total sex-chromosome disomy by total sum of organophosphate metabolites of men seeking infertility evaluation (n=159).

Metabolite[a]	Adjusted IRRs[b]			
	XX18	YY18	XY18	Total Disomy
∑DMAPs				
T1	1.00	1.00	1.00	1.00
T2	0.88 (0.81, 0.95)	1.29 (1.19, 1.40)	0.88 (0.84, 0.92)	0.95 (0.92, 0.98)
T3	0.65 (0.59, 0.72)	1.00 (0.91, 1.12)	0.77 (0.73, 0.82)	0.78 (0.75, 0.82)
∑DEAPs				
T1	1.00	1.00	1.00	1.00
T2	0.99 (0.90, 1.09)	1.02 (0.92, 1.14)	0.87 (0.82, 0.92)	0.92 (0.88, 0.96)
T3	0.79 (0.73, 0.87)	1.31 (1.20, 1.42)	0.83 (0.79, 0.88)	0.90 (0.87, 0.94)

[a]∑DMAPs Exposure Tertiles: T1=1.49≤X≤23.32 nmol/mL (n=53), T2=23.32<X≤124.41 nmol/mL (n=52), T3=X>124.41 nmol/mL (n=54). ∑DEAPs Exposure Tertiles: T1=1.27≤X≤5.47 nmol/mL (n=53), T2=5.47<X≤23.53 nmol/mL (n=52), T3=X>23.53 nmol/mL (n=54). [b] IRRs were adjusted for specific gravity, age, race, BMI, smoking, total sperm concentration, motility and morphology. Since ∑DMAP and ∑DEAP metabolites are subsets of ∑DAPs and are correlated, the IRRs were also adjusted for ∑DAPs to control for these correlations in the models.

Appendix C – Adjusted IRRs for each Disomy Outcome by DAP Metabolite and 3PBA Exposure.

Adjusted IRRs for XX18, YY18, XY18, and total sex chromosome disomy by quartiles of DMP, DMTP, DMDTP, DEP, and DETP and 3PBA exposure.

Table C.1: Adjusted IRRs (95% CI, p-value) for XX18, YY18, XY18, and total sex-chromosome disomy by Quartiles of DMP (Exposure 1) by 3PBA Quartile (Exposure 2) of men seeking infertility evaluation (n=159).

3PBA Quartile[a]	DMP Quartile[b]	XX18 IRR (95% CI) p-value	YY18 IRR (95% CI) p-value	XY18 IRR (95% CI) p-value	Total Disomy IRR (95% CI) p-value
Q1	Q1	1	1	1	1
	Q2	0.57 (0.46, 0.72) 0.0001	0.67 (0.52, 0.85) 0.0013	0.88 (0.79, 0.99) 0.0337	0.75 (0.68, 0.82) 0.0001
	Q3	0.30 (0.14, 0.63) 0.0016	0.35 (0.16, 0.79) 0.0107	0.45 (0.31, 0.65) 0.0001	0.43 (0.33, 0.58) 0.0001
	Q4	0.66 (0.54, 0.82) 0.0001	0.82 (0.54, 1.23) 0.3276	0.73 (0.65, 0.83) 0.0001	0.70 (0.64, 0.77) 0.0001
Q2	Q1	1	1	1	1
	Q2	0.58 (0.49, 0.69) 0.0001	1.33 (1.08, 1.63) 0.0062	0.70 (0.63, 0.78) 0.0001	0.69 (0.64, 0.75) 0.0001
	Q3	0.51 (0.41, 0.63) 0.0001	1.64 (1.21, 2.21) 0.0013	0.78 (0.68, 0.90) 0.0003	0.67 (0.60, 0.74) 0.0001
	Q4	0.53 (0.43, 0.66) 0.0001	1.90 (1.59, 2.27) 0.0001	0.66 (0.58, 0.75) 0.0001	0.67 (0.61, 0.74) 0.0001
Q3	Q1	1	1	1	1
	Q2	0.93 (0.80, 1.08) 0.3411	0.68 (0.53, 0.88) 0.0036	1.00 (0.92, 1.09) 0.9440	1.13 (1.06, 1.21) 0.0002
	Q3	1.06 (0.92, 1.22) 0.4114	0.89 (0.72, 1.11) 0.3119	1.02 (0.94, 1.10) 0.6644	1.09 (1.02, 1.16) 0.0080
	Q4	0.81 (0.66, 0.99) 0.0478	1.11 (0.87, 1.42) 0.3892	0.83 (0.73, 0.93) 0.0019	0.98 (0.89, 1.07) 0.6644
Q4	Q1	1	1	1	1
	Q2	0.51 (0.40, 0.65) 0.0001	0.68 (0.56, 0.83) 0.0001	0.47 (0.41, 0.54) 0.0001	0.53 (0.48, 0.59) 0.0001
	Q3	0.74 (0.60, 0.90) 0.0030	0.32 (0.23, 0.44) 0.0001	0.78 (0.70, 0.87) 0.0001	0.83 (0.76, 0.91) 0.0001
	Q4	1.04 (0.85, 1.26) 0.7267	0.67 (0.54, 0.83) 0.0003	0.89 (0.79, 0.99) 0.0466	0.92 (0.84, 1.01) 0.0665

[a] 3PBA Exposure Quartiles: Q1=X≤LOD (n=33), Q2=0.10<X≤0.61 ng/mL (n=46), Q3=0.61<X≤0.83 ng/mL (n=39), Q4=X>0.83 ng/mL (n=41). [b] DMP Exposure Quartiles: Q1=X≤LOD (n=68), Q2=0.60<X≤7.95 ng/mL (n=30), Q3=7.95<X≤13.39 ng/mL (n=30), Q4=X>13.39 ng/mL (n=31). IRRs were adjusted for specific gravity, age, race, BMI, smoking, total concentration, motility, and morphology.

Table C.2: Adjusted IRRs (95% CI, p-value) for XX18, YY18, XY18, and total sex-chromosome disomy by Quartiles of DMTP (Exposure 1) by 3PBA Quartile (Exposure 2) of men seeking infertility evaluation (n=159).

3PBA Quartile[a]	DMTP Quartile[b]	XX18 IRR (95% CI) p-value	YY18 IRR (95% CI) p-value	XY18 IRR (95% CI) p-value	Total Disomy IRR (95% CI) p-value
Q1	Q1	1	1	1	1
	Q2	1.74 (1.33, 2.29) 0.0001	0.92 (0.73, 1.16) 0.4988	1.29 (1.15, 1.45) 0.0001	1.29 (1.18, 1.41) 0.0001
	Q3	1.15 (0.76, 1.74) 0.4952	0.65 (0.45, 0.94) 0.0221	1.07 (0.94, 1.23) 0.3037	1.08 (0.97, 1.19) 0.1545
	Q4	1.44 (1.01, 2.05) 0.0430	0.91 (0.69, 1.21) 0.5367	1.23 (1.02, 1.49) 0.0268	1.13 (0.97, 1.30) 0.1095
Q2	Q1	1	1	1	1
	Q2	1.42 (1.12, 1.80) 0.0033	1.02 (0.78, 1.34) 0.8683	0.85 (0.69, 1.05) 0.1373	1.05 (0.89, 1.24) 0.5642
	Q3	1.58 (1.25, 1.99) 0.0001	1.34 (1.03, 1.74) 0.0302	0.67 (0.55, 0.80) 0.0001	0.76 (0.66, 0.88) 0.0002
	Q4	1.17 (0.93, 1.47) 0.1816	1.15 (0.88, 1.47) 0.3005	0.99 (0.79, 1.26) 0.9691	1.31 (1.09, 1.57) 0.0035
Q3	Q1	1	1	1	1
	Q2	0.97 (0.76, 1.24) 0.7940	1.23 (0.96, 1.58) 0.0990	1.66 (1.35, 2.05) 0.0001	1.75 (1.48, 2.06) 0.0001
	Q3	1.29 (1.01, 1.65) 0.0424	1.41 (1.08, 1.84) 0.0126	0.94 (0.79, 1.11) 0.4549	0.95 (0.83, 1.07) 0.3696
	Q4	1.67 (1.31, 2.14) 0.0001	2.24 (1.73, 2.90) 0.0001	1.40 (1.13, 1.75) 0.0025	1.26 (1.06, 1.49) 0.0093
Q4	Q1	1	1	1	1
	Q2	2.74 (2.04, 3.66) 0.0001	0.58 (0.43, 0.77) 0.0002	0.92 (0.75, 1.13) 0.4345	1.12 (0.96, 1.32) 0.1564
	Q3	2.74 (2.03, 3.70) 0.0001	1.73 (1.33, 2.24) 0.0001	0.74 (0.62, 0.88) 0.0006	0.94 (0.83, 1.08) 0.3966
	Q4	2.01 (1.48, 2.73) 0.0001	0.68 (0.53, 0.88) 0.0035	0.68 (0.55, 0.85) 0.0007	0.72 (0.61, 0.86) 0.0002

[a] 3PBA Exposure Quartiles: Q1=X≤LOD (n=33), Q2=0.10<X≤0.61 ng/mL (n=46), Q3=0.61<X≤0.83 ng/mL (n=39), Q4=X>0.83 ng/mL (n=41). [b] DMTP Exposure Quartiles: Q1=X≤LOD (n=30), Q2=0.20<X≤2.21 ng/mL (n=43), Q3=2.21<X≤6.47 ng/mL (n=42), Q4=X>6.47 ng/mL (n=44). IRRs were adjusted for specific gravity, age, race, BMI, smoking, total concentration, motility, and morphology.

Table C.3: Adjusted IRRs (95% CI, p-value) for XX18, YY18, XY18, and total sex-chromosome disomy by Quartiles of DMDTP (Exposure 1) by 3PBA Quartile (Exposure 2) of men seeking infertility evaluation (n=159).

3PBA Quartile[a]	DMDTP Quartile[b]	XX18 IRR (95% CI) p-value	YY18 IRR (95% CI) p-value	XY18 IRR (95% CI) p-value	Total Disomy IRR (95% CI) p-value
Q1	Q1	1	1	1	1
	Q2	1.89 (1.55, 2.31) 0.0001	1.30 (1.03, 1.64) 0.0262	1.04 (0.89, 1.21) 0.6436	1.46 (1.33, 1.61) 0.0001
	Q3	1.09 (0.75, 1.58) 0.6640	1.28 (0.92, 1.77) 0.1393	0.69 (0.52, 0.91) 0.0079	1.03 (0.87, 1.23) 0.7099
	Q4	1.35 (1.09, 1.67) 0.0066	1.54 (1.26, 1.88) 0.0001	1.06 (0.85, 1.34) 0.5901	1.31 (1.19, 1.44) 0.0001
Q2	Q1	1	1	1	1
	Q2	1.75 (1.13, 2.70) 0.0125	1.05 (0.70, 1.57) 0.8308	1.15 (1.04, 1.27) 0.0077	1.36 (1.11, 1.67) 0.0031
	Q3	0.42 (0.26, 1.67) 0.0003	0.51 (0.32, 0.81) 0.0044	1.41 (1.29, 1.54) 0.0001	0.66 (0.53, 0.82) 0.0002
	Q4	0.93 (0.77, 1.13) 0.4788	0.98 (0.81, 1.18) 0.8015	1.18 (1.06, 1.32) 0.0022	1.09 (1.00, 1.19) 0.0622
Q3	Q1	1	1	1	1
	Q2	3.35 (2.15, 5.20) 0.0001	1.66 (1.09, 2.51) 0.0173	1.83 (1.58, 2.11) 0.0001	1.96 (1.59, 2.40) 0.0001
	Q3	1.07 (0.70, 1.64) 0.7392	1.53 (1.03, 2.28) 0.0345	1.54 (1.37, 1.73) 0.0001	1.07 (0.88, 1.30) 0.5208
	Q4	1.18 (0.99, 1.40) 0.0609	1.39 (1.16, 1.66) 0.0003	0.86 (0.72, 1.02) 0.0916	1.17 (1.08, 1.26) 0.0001
Q4	Q1	1	1	1	1
	Q2	1.93 (1.24, 3.02) 0.0039	1.11 (0.74, 1.69) 0.6120	1.01 (0.84, 1.22) 0.9035	1.67 (1.36, 2.05) 0.0001
	Q3	0.57 (0.36, 0.88) 0.0116	0.78 (0.51, 1.18) 0.2350	0.82 (0.72, 0.93) 0.0015	0.87 (0.71, 1.06) 0.1666
	Q4	0.65 (0.47, 0.90) 0.0087	0.65 (0.49, 0.86) 0.0029	0.76 (0.64, 0.91) 0.0021	0.95 (0.83, 1.07) 0.3859

[a] 3PBA Exposure Quartiles: Q1=X≤LOD (n=33), Q2=0.10<X≤0.61 ng/mL (n=46), Q3=0.61<X≤0.83 ng/mL (n=39), Q4=X>0.83 ng/mL (n=41). [b] DMDTP Exposure Quartiles: Q1=X≤LOD (n=69), Q2=0.10<X≤0.73 ng/mL (n=30), Q3=0.73<X≤1.86 ng/mL (n=30), Q4=X>1.86 ng/mL (n=30). IRRs were adjusted for specific gravity, age, race, BMI, smoking, total concentration, motility, and morphology.

Table C.4: Adjusted IRRs (95% CI, p-value) for XX18, YY18, XY18, and total sex-chromosome disomy by Quartiles of DEP (Exposure 1) by 3PBA Quartile (Exposure 2) of men seeking infertility evaluation (n=159).

3PBA Quartile[a]	DEP Quartile[b]	XX18 IRR (95% CI) p-value	YY18 IRR (95% CI) p-value	XY18 IRR (95% CI) p-value	Total Disomy IRR (95% CI) p-value
Q1	Q1	1	1	1	1
	Q2	1.33 (1.09, 1.63) 0.0044	1.25 (1.03, 2.53) 0.0224	1.38 (1.24, 1.54) 0.0001	1.33 (1.23, 1.45) 0.0001
	Q3	1.87 (1.53, 2.29) 0.0001	1.11 (0.88, 1.41) 0.3763	1.10 (0.97, 1.26) 0.1386	1.24 (1.13, 1.37) 0.0001
	Q4	1.44 (1.18, 1.75) 0.0003	0.90 (0.73, 1.11) 0.3175	0.97 (0.86, 1.09) 0.5993	1.03 (0.94, 1.13) 0.5218
Q2	Q1	1	1	1	1
	Q2	1.13 (0.84, 1.54) 0.4220	1.08 (0.78, 1.49) 0.6615	1.20 (1.00, 1.45) 0.0535	1.16 (1.00, 1.33) 0.0462
	Q3	1.93 (1.43, 2.60) 0.0001	1.12 (0.81, 1.54) 0.4892	0.82 (0.68, 0.99) 0.0458	1.07 (0.93, 1.23) 0.3513
	Q4	0.55 (0.41, 0.75) 0.0001	0.47 (0.34, 0.66) 0.0001	0.71 (0.59, 0.86) 0.0005	0.62 (0.53, 0.71) 0.0001
Q3	Q1	1	1	1	1
	Q2	1.17 (0.89, 1.53) 0.2699	2.10 (1.55, 2.85) 0.0001	1.78 (1.52, 2.09) 0.0001	1.68 (1.48, 1.91) 0.0001
	Q3	1.12 (0.84, 1.49) 0.4376	1.61 (1.18, 2.19) 0.0027	0.74 (0.62, 0.88) 0.0007	0.95 (0.84, 1.09) 0.4941
	Q4	0.90 (0.68, 1.19) 0.4543	1.43 (1.04, 1.98) 0.0301	0.82 (0.69, 0.99) 0.0376	0.92 (0.80, 1.06) 0.2381
Q4	Q1	1	1	1	1
	Q2	0.97 (0.72, 1.31) 0.8582	1.43 (1.04, 1.95) 0.0264	1.37 (1.15, 1.64) 0.0004	1.27 (1.11, 1.46) 0.0005
	Q3	1.92 (1.40, 2.61) 0.0001	1.05 (0.75, 1.47) 0.7846	1.11 (0.92, 1.34) 0.2733	1.24 (1.07, 1.43) 0.0035
	Q4	0.42 (0.30, 0.58) 0.0001	0.61 (0.43, 0.86) 0.0046	0.64 (0.52, 0.78) 0.0001	0.57 (0.49, 0.67) 0.0001

[a] 3PBA Exposure Quartiles: Q1=X≤LOD (n=33), Q2=0.10<X≤0.61 ng/mL (n=46), Q3=0.61<X≤0.83 ng/mL (n=39), Q4=X>0.83 ng/mL (n=41. [b] DEP Exposure Quartiles: Q1=X≤LOD (n=58), Q2=0.20<X≤1.46 ng/mL (n=34), Q3=1.46<X≤3.96 ng/mL (n=33), Q4=X>3.96 ng/mL (n=34). IRRs were adjusted for specific gravity, age, race, BMI, smoking, total concentration, motility, and morphology.

Table C.5: Adjusted IRRs (95% CI, p-value) for XX18, YY18, XY18, and total sex-chromosome disomy by Quartiles of DETP (Exposure 1) by 3PBA Quartile (Exposure 2) of men seeking infertility evaluation (n=159).

3PBA Quartile[a]	DETP Quartile[b]	XX18 IRR (95% CI) p-value	YY18 IRR (95% CI) p-value	XY18 IRR (95% CI) p-value	Total Disomy IRR (95% CI) p-value
Q1	Q1	1	1	1	1
	Q2	1.54 (1.24, 1.91) 0.0001	1.27 (1.03, 1.58) 0.0283	0.86 (0.75, 0.98) 0.0228	1.04 (0.94, 1.15) 0.4495
	Q3	2.62 (2.15, 3.20) 0.0001	3.05 (2.49, 3.73) 0.0001	1.64 (1.46, 1.84) 0.0001	2.00 (1.83, 2.19) 0.0001
	Q4	1.50 (1.20, 1.88) 0.0003	1.33 (1.04, 1.70) 0.0235	0.74 (0.65, 0.85) 0.0001	0.96 (0.87, 1.07) 0.4590
Q2	Q1	1	1	1	1
	Q2	0.37 (0.28, 0.50) 0.0001	0.48 (0.36, 0.65) 0.0001	0.35 (0.29, 0.42) 0.0001	0.39 (0.34, 0.44) 0.0001
	Q3	2.58 (1.96, 3.41) 0.0001	4.13 (3.10, 5.49) 0.0001	1.71 (1.43, 2.06) 0.0001	2.31 (2.02, 2.64) 0.0001
	Q4	0.72 (0.58, 0.90) 0.0036	0.94 (0.75, 1.18) 0.6130	0.72 (0.63, 0.82) 0.0001	0.76 (0.69, 0.84) 0.0001
Q3	Q1	1	1	1	1
	Q2	0.59 (0.46, 0.77) 0.0001	0.83 (0.63, 1.11) 0.2117	0.56 (0.48, 0.66) 0.0001	0.60 (0.53, 0.68) 0.0001
	Q3	1.41 (1.06, 1.86) 0.0171	4.11 (3.01, 5.61) 0.0001	1.73 (1.45, 2.06) 0.0001	1.89 (1.65, 2.16) 0.0001
	Q4	0.76 (0.63, 0.91) 0.0025	2.37 (1.94, 2.89) 0.0001	0.67 (0.61, 0.75) 0.0001	0.87 (0.80, 0.95) 0.0014
Q4	Q1	1	1	1	1
	Q2	0.67 (0.49, 0.92) 0.0130	0.31 (0.23, 0.42) 0.0001	0.59 (0.49, 0.72) 0.0001	0.53 (0.46, 0.61) 0.0001
	Q3	1.38 (0.95, 2.01) 0.0898	1.51 (1.04, 2.18) 0.0300	1.24 (0.99, 1.57) 0.0656	1.28 (1.08, 1.52) 0.0051
	Q4	0.85 (0.69, 1.07) 0.1586	0.70 (0.57, 0.87) 0.0012	0.92 (0.81, 1.05) 0.2403	0.84 (0.76, 0.93) 0.0006

[a] 3PBA Exposure Quartiles: Q1=X≤LOD (n=33), Q2=0.10<X≤0.61 ng/mL (n=46), Q3=0.61<X≤0.83 ng/mL (n=39), Q4=X>0.83 ng/mL (n=41). [b] DETP Exposure Quartiles: Q1=X≤LOD (n=45), Q2=0.10<X≤0.62 ng/mL (n=39), Q3=0.62<X≤1.51 ng/mL (n=37), Q4=X>1.51 ng/mL (n=38). IRRs were adjusted for specific gravity, age, race, BMI, smoking, total concentration, motility, and morphology.

Adjusted IRRs for XX18, YY18, XY18, and total sex chromosome disomy by exposure group of DEDTP and 3PBA exposure.

Table C.6: Adjusted IRRs (95% CI, p-value) for XX18, YY18, XY18, and total sex-chromosome disomy by Group of DEDTP (Exposure 1) by 3PBA Quartile (Exposure 2) of men seeking infertility evaluation (n=159).

3PBA Quartile[a]	DEDTP Group[b]	XX18 IRR (95% CI) p-value	YY18 IRR (95% CI) p-value	XY18 IRR (95% CI) p-value	Total Disomy IRR (95% CI) p-value
Q1	G1	1	1	1	1
	G2	1.82 (1.50, 2.20) 0.0001	1.03 (0.80, 1.32) 0.8417	1.23 (1.08, 1.40) 0.0023	1.31 (1.19, 1.45) 0.0001
Q2	G1	1	1	1	1
	G2	0.25 (0.17, 0.39) 0.0001	0.60 (0.46, 0.80) 0.0002	0.51 (0.41, 0.63) 0.0001	0.49 (0.42, 0.57) 0.0001
Q3	G1	1	1	1	1
	G2	1.19 (0.97, 1.44) 0.0889	0.87 (0.70, 1.09) 0.2387	0.87 (0.77, 0.99) 0.0336	0.92 (0.84, 1.01) 0.0954
Q4	G1	1	1	1	1
	G2	1.21 (1.01, 1.46) 0.0407	0.97 (0.79, 1.18) 0.7367	0.91 (0.82, 1.03) 0.1248	0.98 (0.90, 1.07) 0.6335

[a] 3PBA Exposure Quartiles: Q1=X≤LOD (n=33), Q2=0.10<X≤0.61 ng/mL (n=46), Q3=0.61<X≤0.83 ng/mL (n=39), Q4=X>0.83 ng/mL (n=41). [b] DEDTP Exposure Group: G1=X≤LOD (n=143); G2=X>0.10 ng/mL (n=16).
IRRs were adjusted for specific gravity, age, race, BMI, smoking, total concentration, motility, and morphology.

Adjusted IRRs for XX18, YY18, XY18, and total sex chromosome disomy by tertiles of ∑DAPs and 3PBA exposure.

Table C.7: Adjusted IRRs (95% CI, p-value) for XX18, YY18, XY18, and total sex-chromosome disomy by Tertiles of DAPs (Exposure 1) by 3PBA Tertiles (Exposure 2) of men seeking infertility evaluation (n=159).

3PBA Tertile[a]	DAPs Tertile[b]	XX18 IRR (95% CI) p-value	YY18 IRR (95% CI) p-value	XY18 IRR (95% CI) p-value	Total Disomy IRR (95% CI) p-value
T1	T1	1	1	1	1
	T2	1.60 (1.36, 1.89) 0.0001	0.61 (0.46, 0.81) 0.0006	1.02 (0.93, 1.12) 0.6800	1.10 (1.02, 1.18) 0.0145
	T3	1.03 (0.81, 1.31) 0.8122	0.81 (0.66, 1.01) 0.0595	0.94 (0.83, 1.07) 0.3540	0.92 (0.83, 1.02) 0.1129
T2	T1	1	1	1	1
	T2	1.09 (0.93, 1.26) 0.2943	1.41 (1.23, 1.61) 0.0001	0.78 (0.71, 0.86) 0.0001	1.00 (0.93, 1.07) 0.8651
	T3	0.69 (0.59, 0.80) 0.0001	1.21 (1.03, 1.42) 0.0173	0.68 (0.62, 0.74) 0.0001	0.75 (0.70, 0.80) 0.0001
T3	T1	1	1	1	1
	T2	0.81 (0.72, 0.91) 0.0006	1.85 (1.57, 2.19) 0.0001	0.96 (0.89, 1.02) 0.2065	1.01(0.96, 1.07) 0.7037
	T3	0.89 (0.79, 1.01) 0.0613	1.13 (0.94, 1.36) 0.1854	0.95 (0.88, 1.02) 0.1316	0.99 (0.94, 1.05) 0.7681

[a] 3PBA Exposure Tertiles: T1=X≤LOD (n=33), T2=0.10<X≤0.69 (n=68), T3=X>0.69 (n=58). [b] ∑DAPs Exposure Tertiles: T1=2.76≤X≤35.00 (n=53), T2=35.00<X≤155.00 (n=52), T3=X>155.01 (n=54). ∑DAPs is the sum of all six individual metabolites.

IRRs were adjusted for specific gravity, age, race, BMI, smoking, total concentration, motility, and morphology.

IRRs were adjusted for specific gravity, age, race, BMI, smoking, total concentration, motility, and morphology.

Appendix D – Graphs of Adjusted IRRs for each Disomy Outcome by DAP Metabolites and 3PBA Exposure.

To further explore the interactions between OP and PYR metabolites, when the interaction was significant in the model (Table 4.4), graphs of the adjusted IRRs and 95% confidence intervals were depicted for each disomy outcome by quartiles of DAP metabolite (DMP, DMTP, DMDTP, DEP, DETP) by 3PBA quartiles (Figures D.1-D.11).

Graphs of the adjusted IRRs and 95% confidence intervals were also depicted for each disomy outcome by group of DEDTP metabolite by 3PBA quartiles (Figures D.12-D.15) and for each disomy outcome by tertiles of ∑DAP metabolite by 3PBA tertiles (Figures D.16-D.17).

Since statistically significant interactions between all DAP metabolites and 3PBA were consistently observed for total sex chromosome disomy, graphs of the adjusted IRRs and 95% confidence intervals have been further evaluated and discussed in Chapter 4.

Figure D.1. Adjusted IRRs (95% CI) for XX18 by Quartiles of DMP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

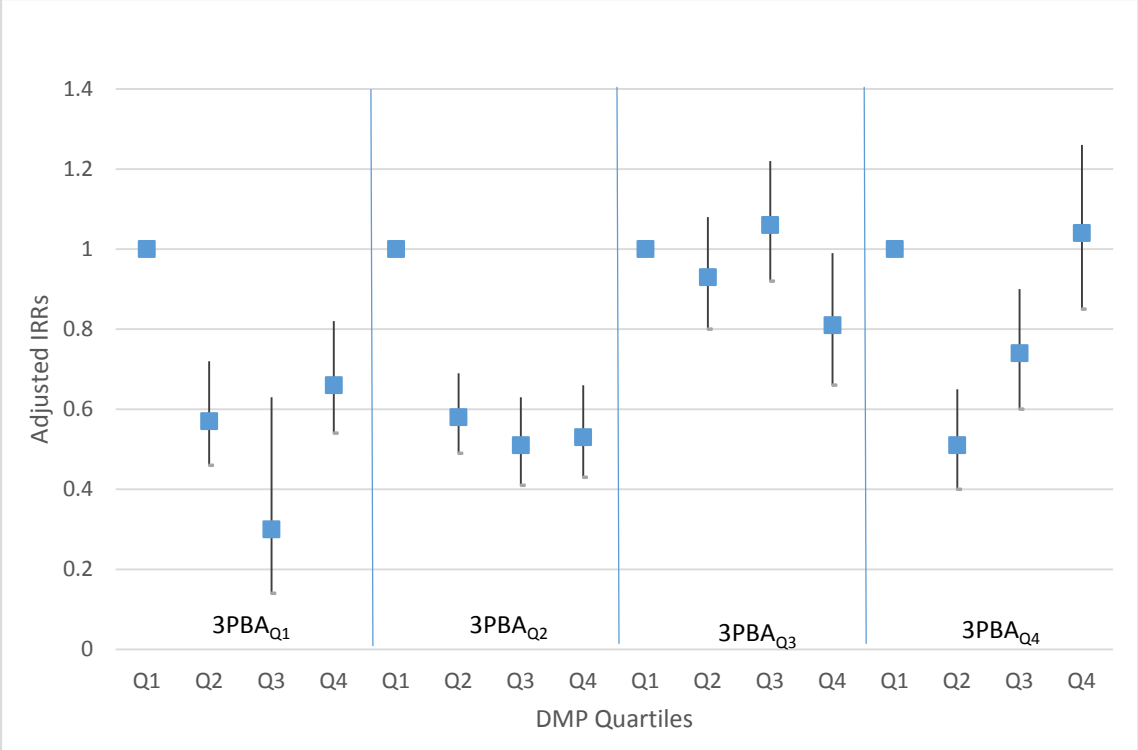


Figure D.2. Adjusted IRRs (95% CI) for XY18 by Quartiles of DMP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

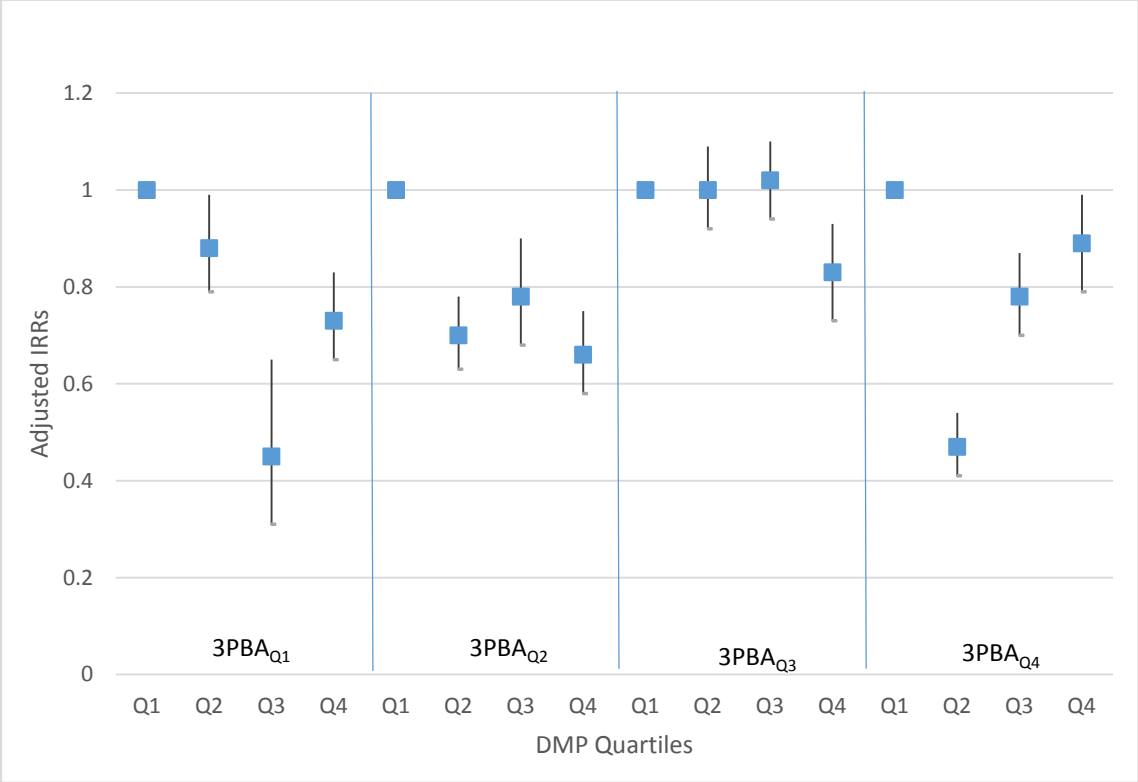


Figure D.3. Adjusted IRRs (95% CI) for XY18 by Quartiles of DTMP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

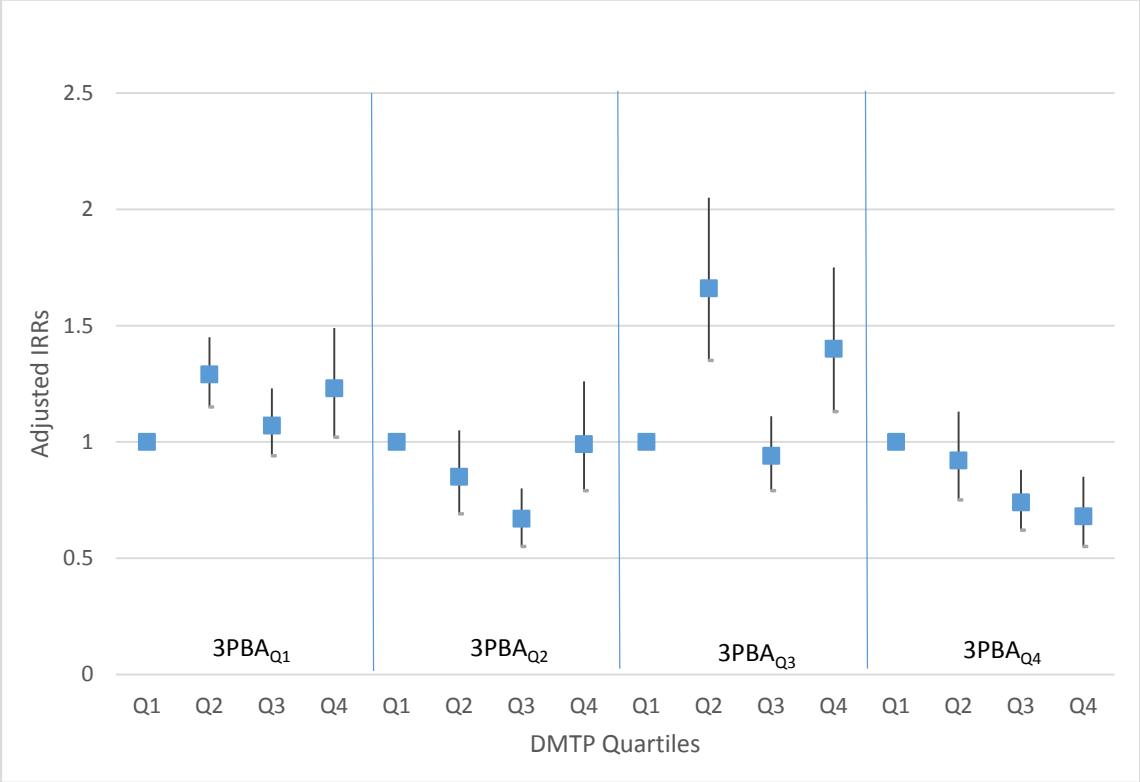


Figure D.4. Adjusted IRRs (95% CI) for XX18 by Quartiles of DMDTP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

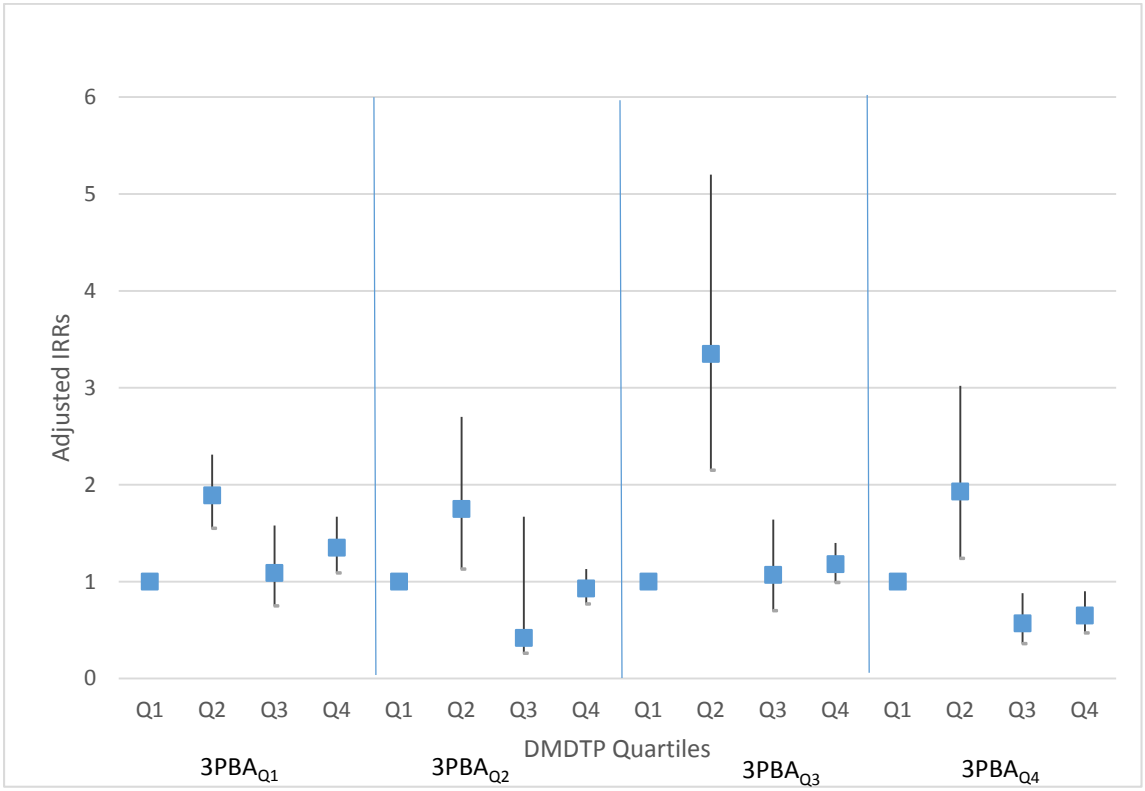


Figure D.5. Adjusted IRRs (95% CI) for YY18 by Quartiles of DMDTP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

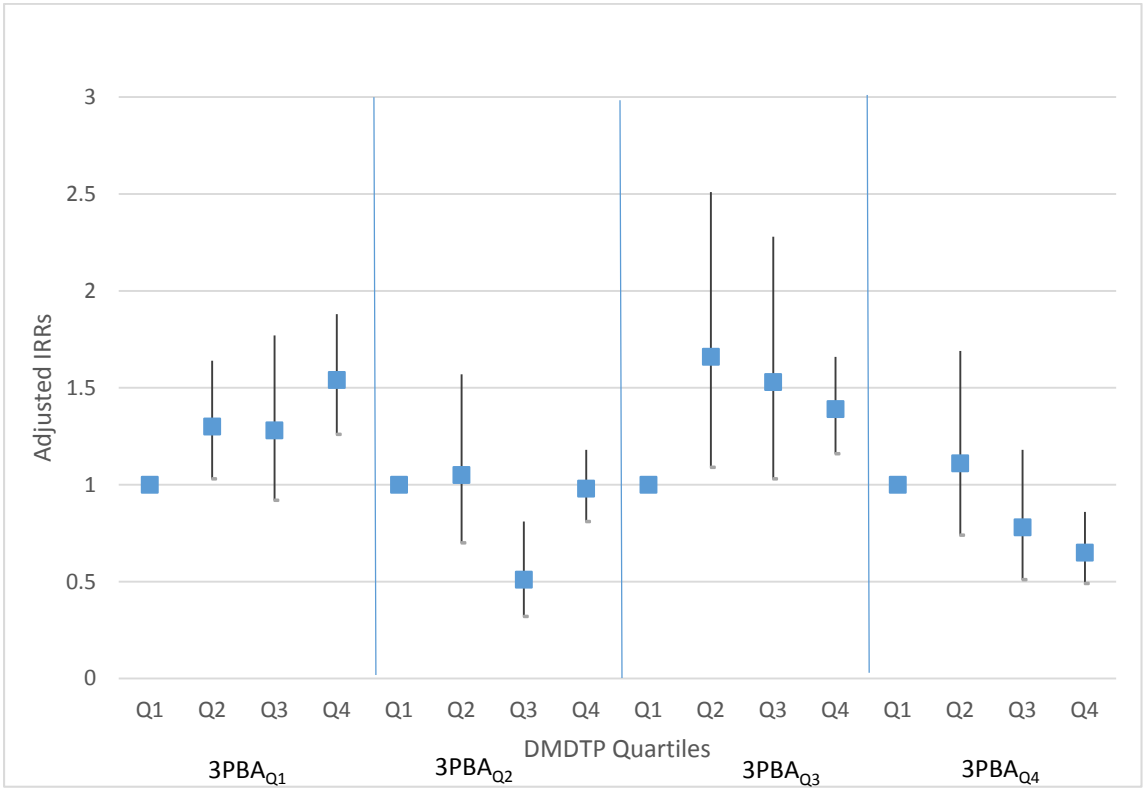


Figure D.6. Adjusted IRRs (95% CI) for XX18 by Quartiles of DEP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

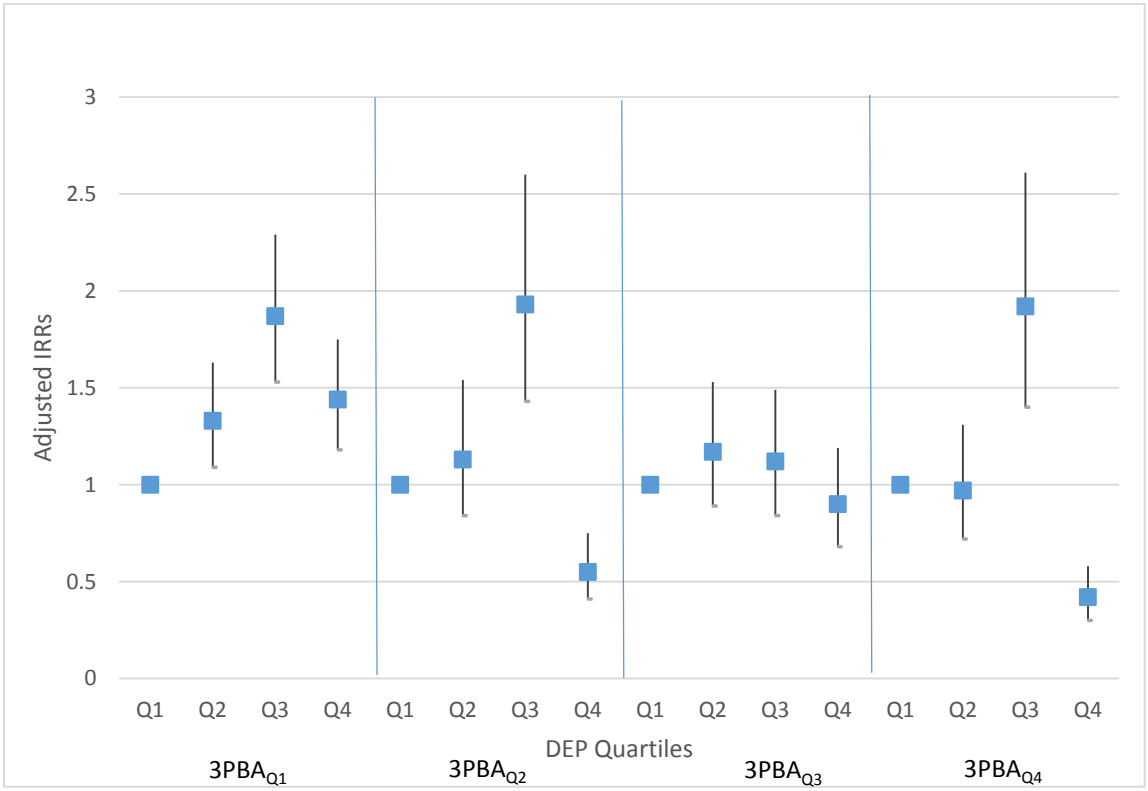


Figure D.7. Adjusted IRRs (95% CI) for YY18 by Quartiles of DEP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

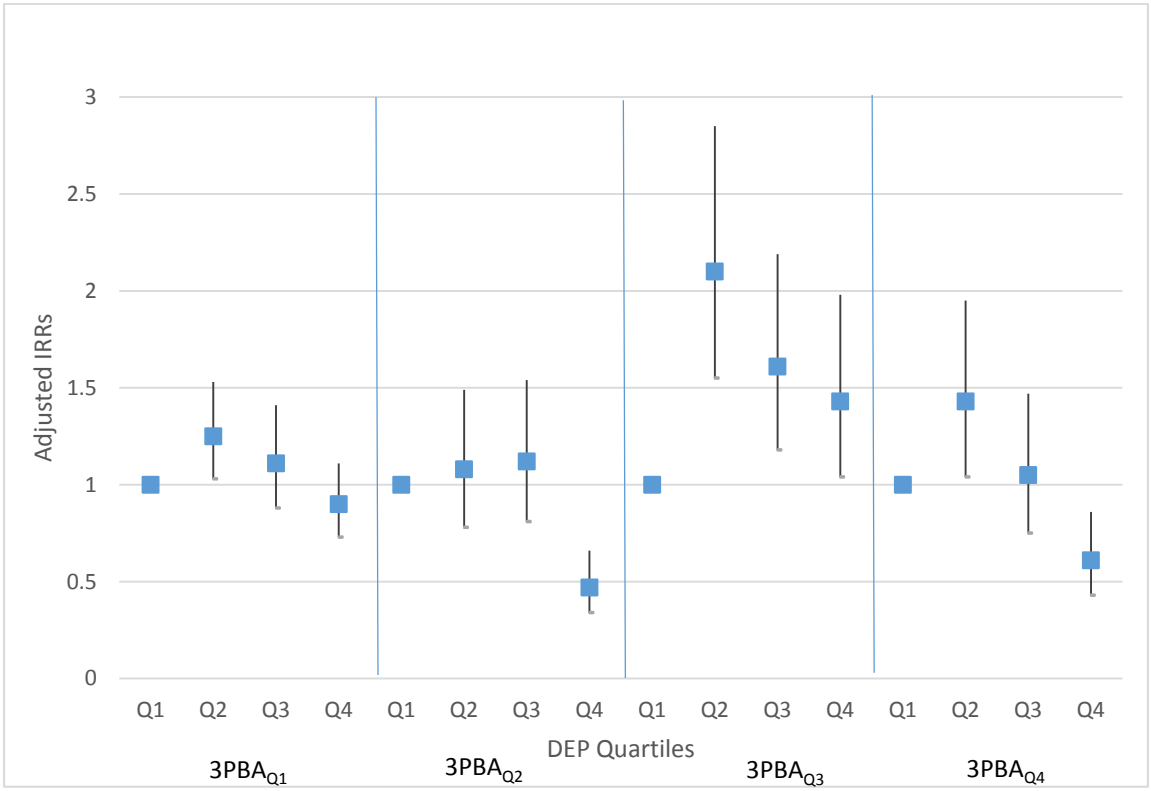


Figure D.8. Adjusted IRRs (95% CI) for XY18 by Quartiles of DEP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

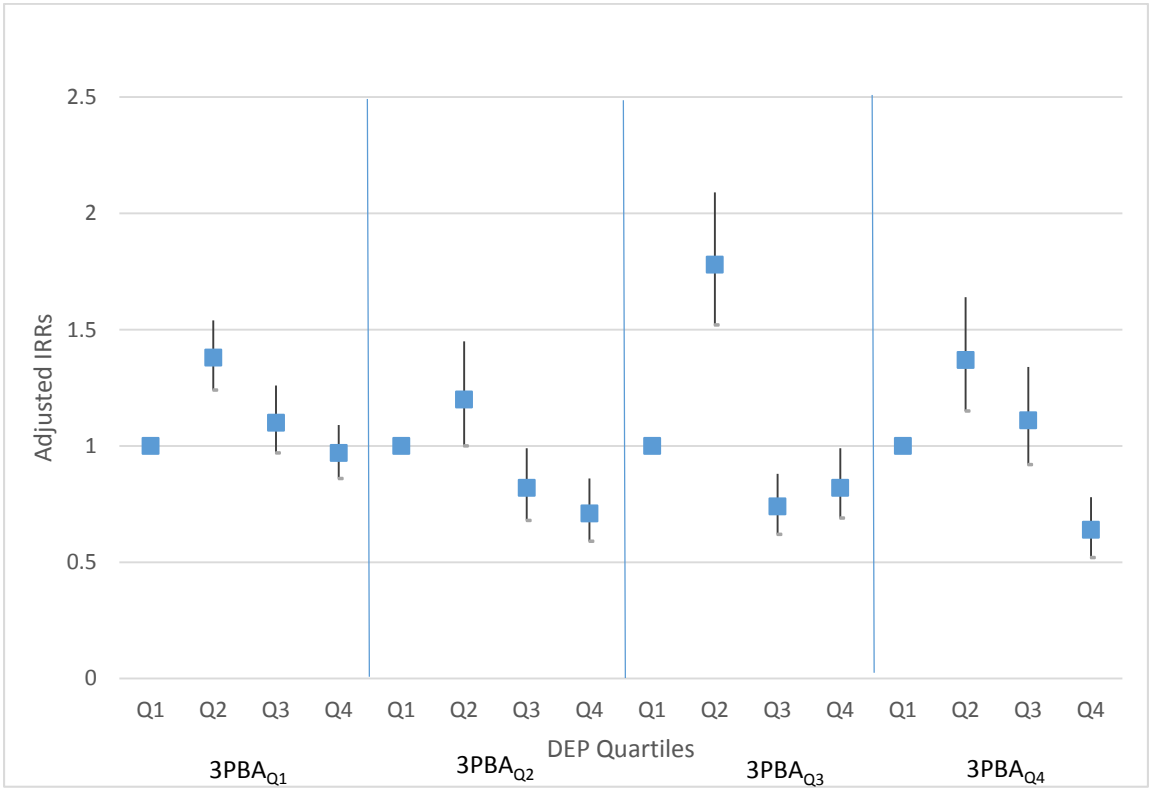


Figure D.9. Adjusted IRRs (95% CI) for XX18 by Quartiles of DETP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

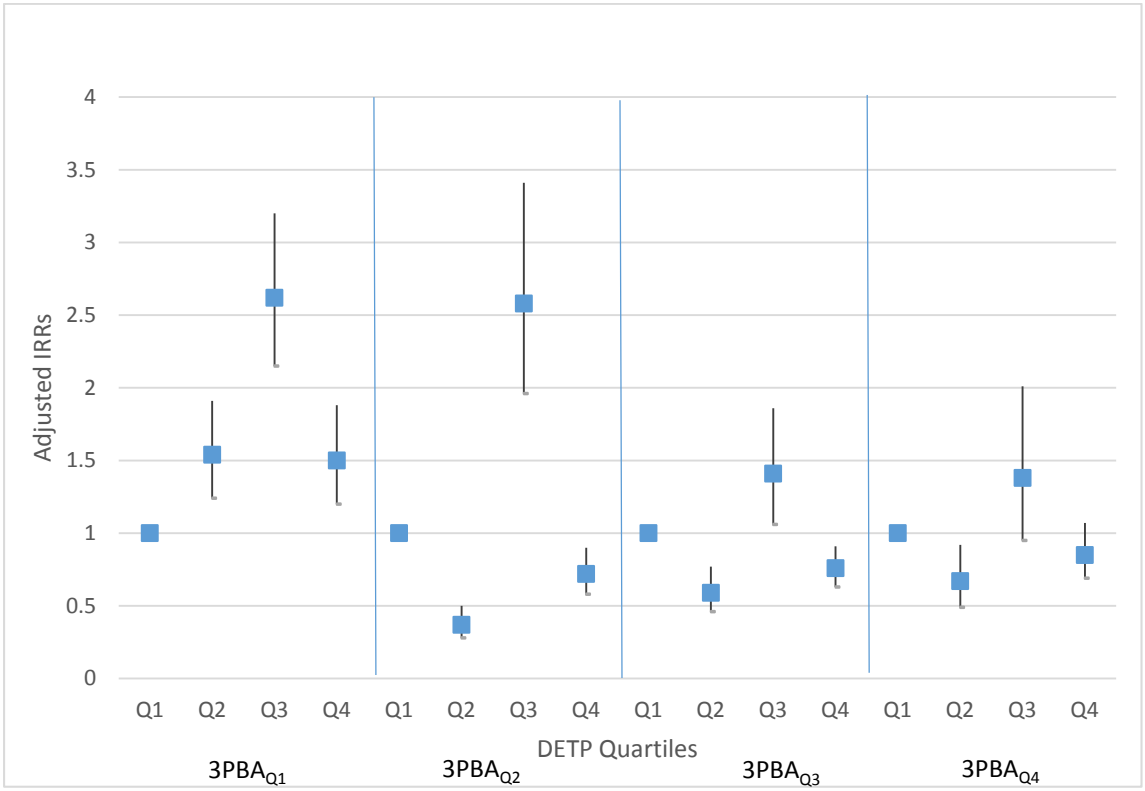


Figure D.10. Adjusted IRRs (95% CI) for YY18 by Quartiles of DETP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

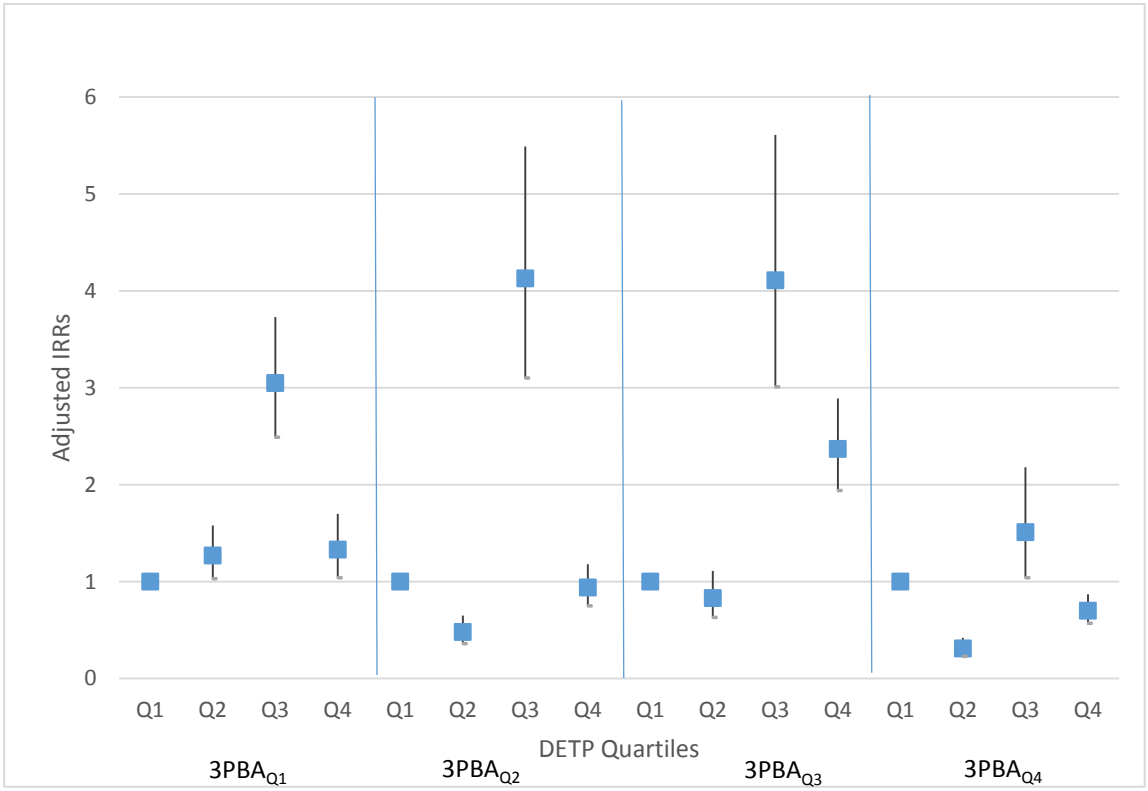


Figure D.11. Adjusted IRRs (95% CI) for XY18 by Quartiles of DETP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

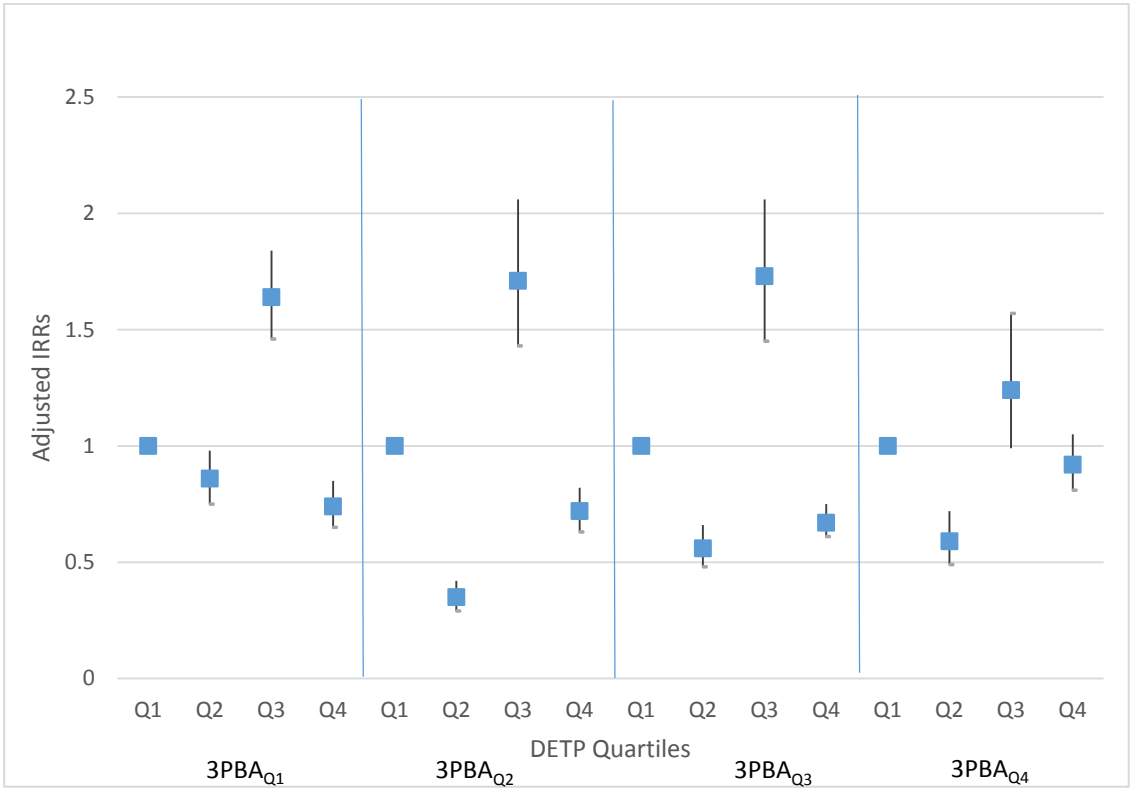


Figure D.12. Adjusted IRRs (95% CI) for XX18 by Group of DEDTP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

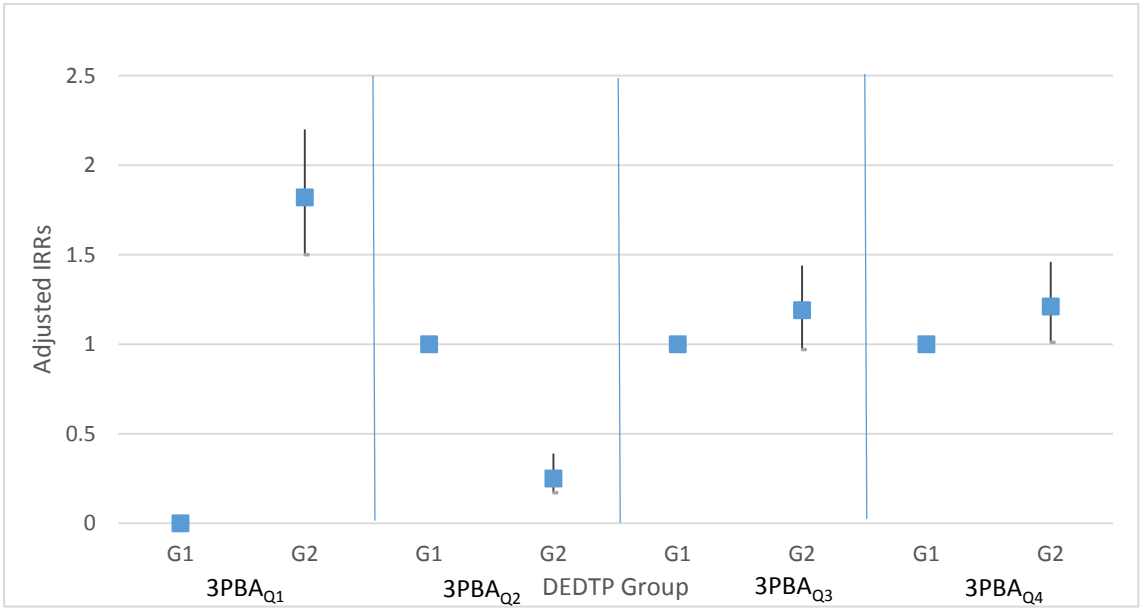


Figure D.13. Adjusted IRRs (95% CI) for YY18 by Group of DEDTP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

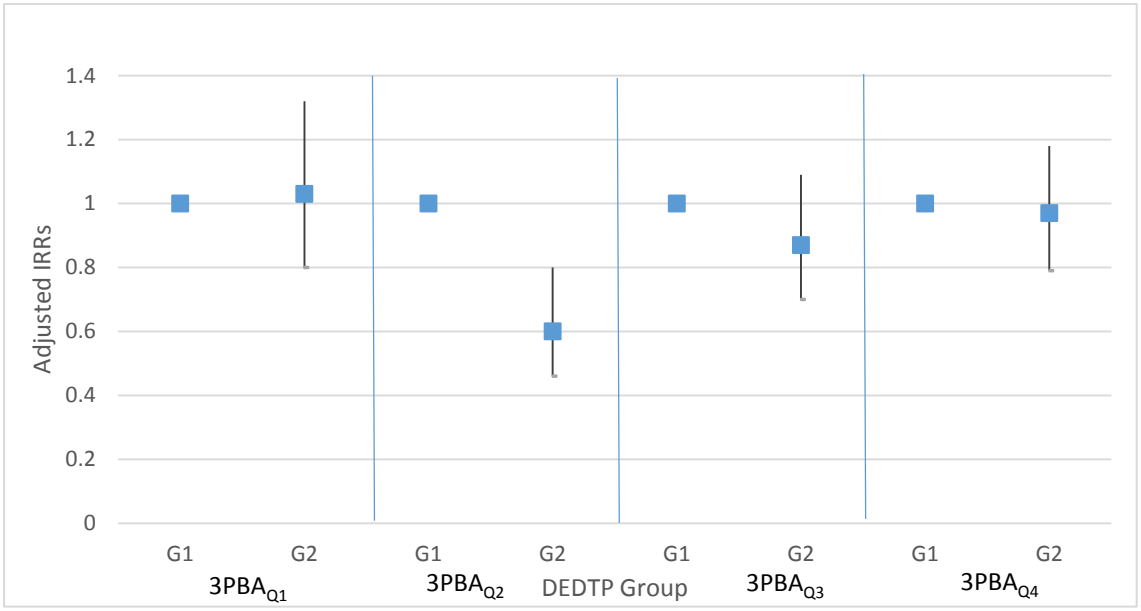


Figure D.14. Adjusted IRRs (95% CI) for XY18 by Group of DEDTP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).

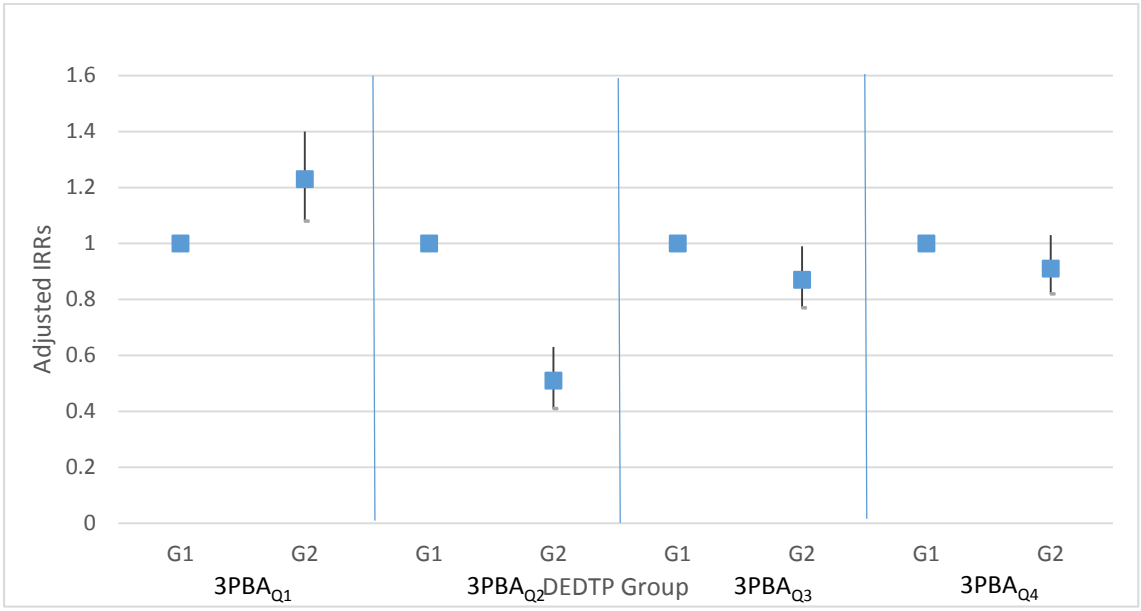
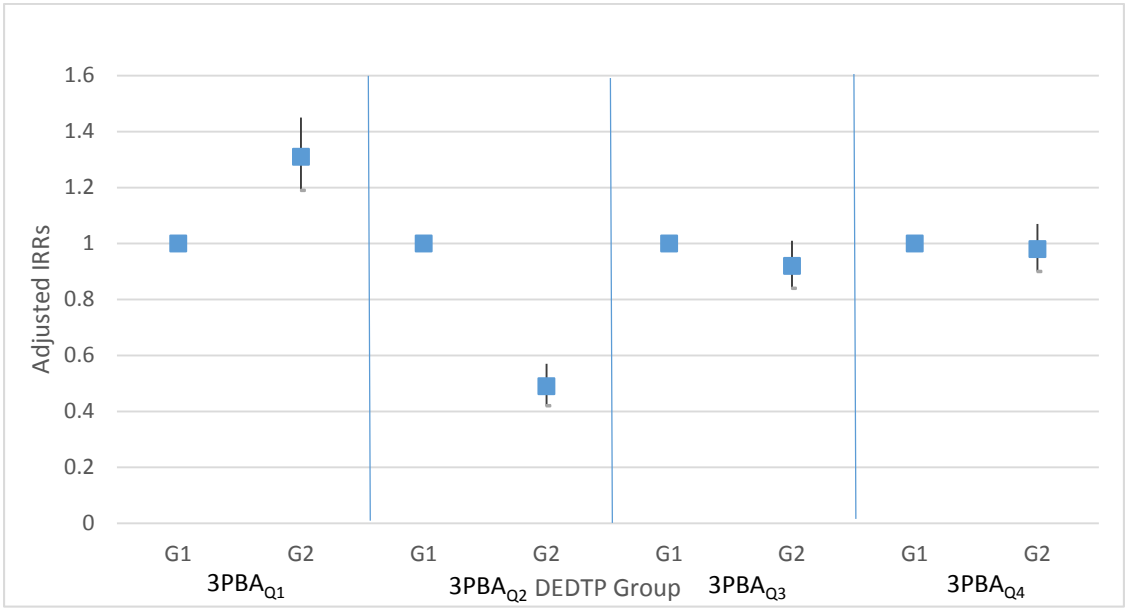


Figure D.15. Adjusted IRRs (95% CI) for Total Sex Chromosome Disomy by Group of DEDTP (Exposure 1 changing) by 3PBA Quartiles (Exposure 2 constant).



*Tests for trend for adjusted IRRs (95% CI) for total sex chromosome disomy by group of DEDTP by 3PBA quartiles were not explored further or discussed in Chapter 4 because there are only two exposure categories of DEDTP metabolite.

Figure D.16. Adjusted IRRs (95% CI) for XX18 by Tertiles of ∑DAPs (Exposure 1 changing) by 3PBA Tertiles (Exposure 2 constant).

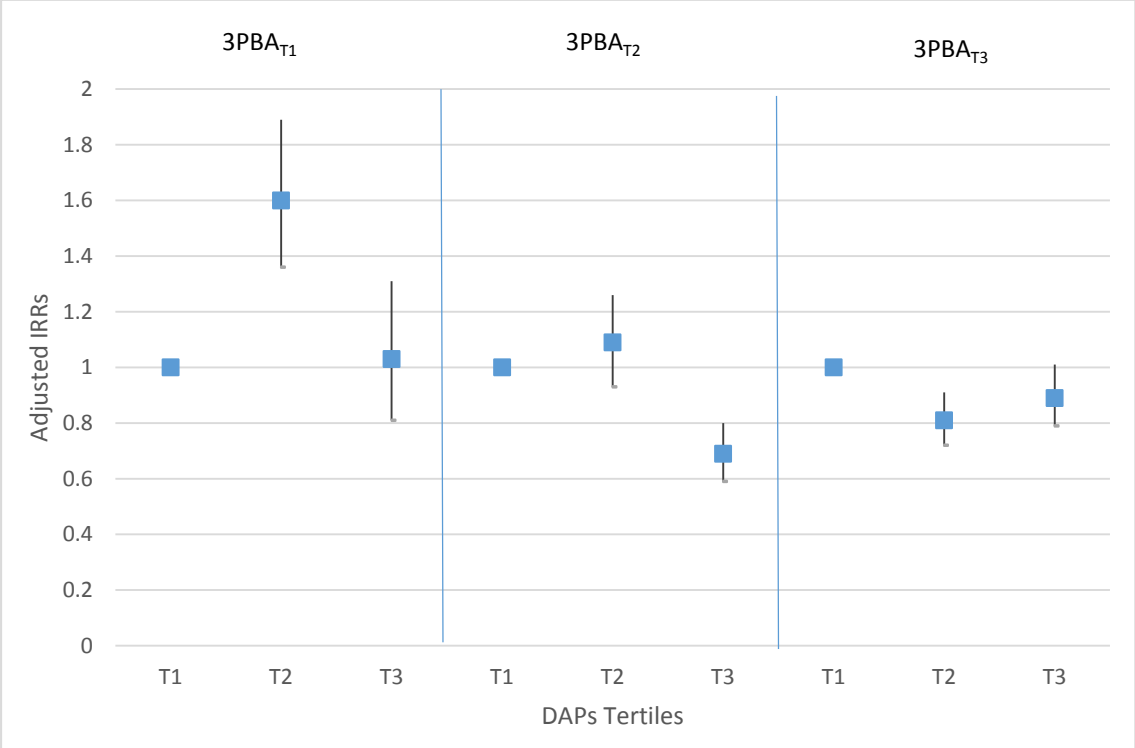
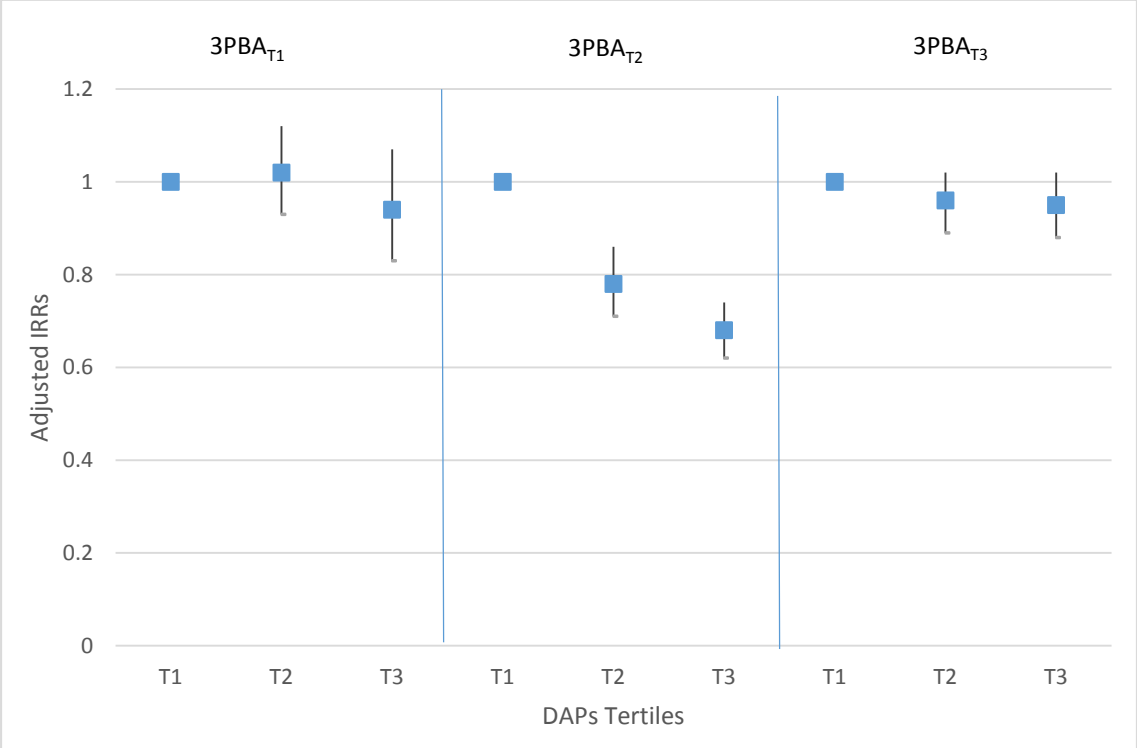


Figure D.17. Adjusted IRRs (95% CI) for XY18 by Tertiles of ∑DAPs (Exposure 1 changing) by 3PBA Tertiles (Exposure 2 constant).



Appendix E – Graphical Representation of a Poisson Distribution/Regression.

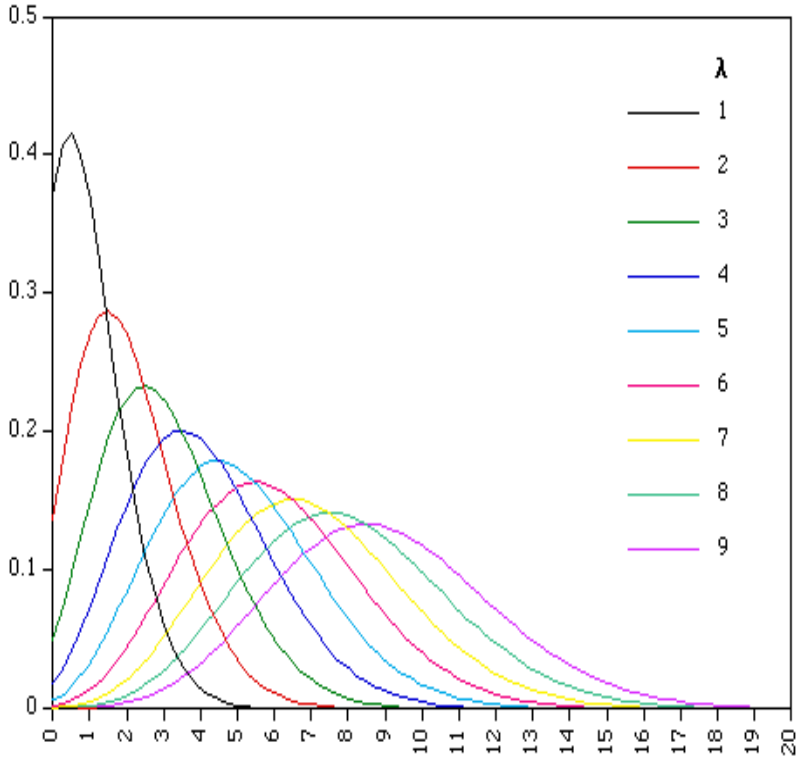
A poisson distribution/regression include the following basic characteristics:

1) it is utilized to model count data and rates;

2) the response or outcome variable (Y) is a count variable;

3) it is utilized for low frequency or rare event, where 0 is a likely number to obtain; and,

4) the basic poisson distribution is skewed toward the right (i.e., not symmetrical).



(A Star Math and Physics Tuition & Course Materials, Greenwich, London)