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ΜΕΤΑΠΤΥΧΙΑΚΗ ΕΡΓΑΣΙΑ

Σχέση της έκθεσης σε επίμονους οργανικούς ρύπους στην εγκυμοσύνη και εμφάνιση δεικτών γενοτοξικότητας σε παιδιά προσχολικής ηλικίας

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Μεταπτυχιακή Διατριβή που υποβάλλεται
στο καθηγητικό σώμα για την μερική εκπλήρωση των υποχρεώσεων απόκτησης του μεταπτυχιακού τίτλου του Μεταπτυχιακού Προγράμματος «Διοίκηση Υπηρεσιών Υγείας» του Τμήματος Ιατρικής του Πανεπιστημίου Κρήτης
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Ευχαριστίες

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Εισαγωγή: Οι επιδράσεις των περιβαλλοντικών ρύπων στην υγεία των παιδιών αποτελούν μείζον θέμα δημόσιας υγείας. Οι ¨επίμονοι¨ οργανικοί ρύποι (POPs) βιοσυσσωρεύονται μέσω της τροφικής αλυσίδας ενώ η έκθεση στους ρύπους αυτούς στα πρώτα στάδια της ζωής μπορεί να σχετίζεται με την εμφάνιση καρκίνων σε ενήλικες και παιδιά. Οι μικροπυρήνες (ΜΝ) είναι πολύ μικροί πυρήνες που προέρχονται από βλάβες του γενετικού υλικού και χρησιμοποιούνται ως δείκτες προκαρκινικών γεγονότων. Η παρουσία τους έχει συσχετιστεί με καρκίνους σε ενήλικες ενώ τα δεδομένα για τη συχνότητα των μικροπυρήνων στα παιδιά είναι λιγοστά. Η παρούσα μελέτη είχε ως στόχο για πρώτη φορά την διερεύνηση της επίδρασης της ενδομήτριας έκθεσης σε επίμονους οργανικούς ρύπους στην συχνότητα εμφάνισης των μικροπυρήνων στα Τ λεμφοκύτταρα σε παιδιά προσχολικής ηλικίας στην κοόρτη μητέρας-παιδιού Κρήτης (Μελέτη Ρέα) (n=328).

Μέθοδοι: Οι συγκεντρώσεις διαφόρων πολυχλωριομένων διφαινυλίων (PCBs), του δί-χλωρο-δι-φαινυλ-τριχλωροαιθανίου (DDT)/ δι-χλωρο-δι-φαινιλ-διχλωροεθυλενίου (DDE) και του εξαχλωροβενζιλιου (HCB) μετρήθηκαν με αέριο χρωματογραφία- φασματομετρία μαζας σε δείγματα ορού από το πρώτο τρίμηνο της εγκυμοσύνης. Μετρήσαμε τη συχνότητα εμφάνισης των μικροπυρήνων ανά 1000 διπλασιασμένα κύτταρα στα Τ λεμφοκύτταρα του περιφερικού αίματος των παιδιών στην ηλικία 4 ετών με τη μέθοδο της κυτταροκινητικής παρεμπόδισης μικροπυρήνων. Χρησιμοποιήθηκαν μοντέλα πολλαπλής αρνητικής διονυμικής παλινδρόμησης για την διερεύνηση των συσχετίσεων μεταξύ της ενδομήτριας έκθεσης σε επίμονους οργανικούς ρύπους και της συχνότητας εμφάνισης των μικροπυρήνων στα παιδιά τεσσάρων ετών.

Αποτελέσματα: Ο γεωμετρικός μέσος όρος για τις συγκεντρώσεις των DDE, HCB και PCBs στις έγκυες ήταν 1960.1 pg/ml, 87.7 pg/ml και 329.9 pg/ml αντίστοιχα. Στα μοντέλα πολυπαραγοντικής ανάλυσης, η αύξηση κατά 10 μονάδες των επιπέδων του DDE σχετίστηκε με αυξημένο κίνδυνο (IRR 1,47 95% CI: 1.05, 2.06). Αντίστοιχα για τα PCBs η

αύξηση των επιπέδων κατά 10 μονάδες σχετίστηκε με αυξημένο κίνδυνο εμφάνισης μικροπυρήνων στα παιδιά (IRR 2.56 95% CI:1.34, 4.91) λαμβάνοντας υπόψη ως συγχυτικούς παράγοντες την ηλικία της μητέρας, την τοκότητα, το φύλλο του παιδιού, το δείκτη μάζας σώματος πριν την εγκυμοσύνη, το εκπαιδευτικό επίπεδο της μητέρας, το παθητικό κάπνισμα, το κάπνισμα κατά την εγκυμοσύνη και την διάρκεια του θηλασμού, τα τριγλυκερίδια και τη χολιστερίνη ορού της μητέρας. Δεν παρατηρήθηκε σχέση ανάμεσα στην ενδομήτρια έκθεση στο HCB και την εμφάνιση μικροπυρήνων κατά την παιδική ηλικία. Η έκθεση στο DDT και τα dioxin-like PCBs κατά την εγκυμοσύνη και ο κίνδυνος εμφάνισης μικροπυρήνων στα παιδιά προσχολικής ηλικίας εμφάνισε γραμμική σχέση δόσης αποτελέσματος.

Συμπεράσματα: Η ενδομήτρια έκθεση σε DDE και PCBs σχετίζεται με την εμφάνιση μικροπυρήνων στα παιδιά στην ηλικία των 4 ετών. Οι επίμονοι οργανικοί ρύποι ενδεχομένως να προκαλούν γενοτοξικά φαινόμενα σε μικρές ηλικίες. Αυτή είναι η πρώτη μελέτη που συνδέει την εμφάνιση μικροπυρήνων στα παιδιά με την ενδομήτρια έκθεση σε DDE και PCBs και απαιτούνται περαιτέρω μελέτες που θα επιβεβαιώσουν αυτά τα αποτελέσματα. Στα επόμενα στάδια της μελέτης θα εκτιμηθεί ο κίνδυνος εμφάνισης μικροπυρήνων σε σχέση με την πρώιμη έκθεση σε περιβαλλοντικούς ρύπους σε μεγαλύτερες ηλικίες παιδιών. Περαιτέρω μελέτες είναι απαραίτητες για να εξετάσουν τους βιολογικούς μηχανισμούς που σχετίζονται με την εμφάνισης μικροπυρήνων μετά από την ενδομήτρια έκθεση σε επίμονους οργανικούς ρύπους και την πιθανή γενοτοξική τους δράση.

Λέξεις κλειδιά: επίμονοι οργανικοί ρύποι, γενοτοξικότητα, εγκυμοσύνη, παιδιά, μικροπυρήνες

Abstract

Title: Persistent organic pollutants during pregnancy and DNA damage in preschool children in Crete

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Introduction: The potential adverse effects of environmental chemicals on children's health and development are a matter of widespread public health concern. Persistent organic pollutants (POPs) bioaccumulate through the food chain and human exposure to POPs has been associated with certain cancers in adults and children. Micronuclei (MN) are extranuclear small nuclei caused by DNA damage, serve as markers of pre-carcinogenic events and have been associated with cancer risk in adults but data on MN frequency in children are limited. Our aim was to investigate for the first time the association between in utero exposure to POPs with MN frequency in lymphocytes of preschool children from the Rhea mother-child cohort in Crete, Greece (n=328).

Methods: Concentrations of several polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT)/dichlorodiphenyldichloroethylene (DDE), and hexachlorobenzene (HCB) were determined in first trimester maternal serum by triple quadrupole mass spectrometry. We used the cytokinesis-block micronucleus assay to assess MN frequencies in 1000 binucleated T-lymphocytes (MNBN) in children at 4 years of age.Negative binomial regression models were used to estimate associations between POP concentrations during pregnancy and MN frequencies in childhood.

Results: Geometric mean DDE, HCB and PCBs serum concentrations in pregnant women were 1960.1 pg/ml, 87.7 pg/ml and 329.9 pg/ml respectively. On multivariable regression analyses, a 10-fold increase in DDE levels in pregnancy was associated with increased risk of micronuclei formation after adjusting for mother's age, parity, child's sex, pre-pregnancy BMI, mother's educational level, passive smoking, smoking during early pregnancy and breastfeeding duration, maternal serum levels of triglycerides and total cholesterol (IRR 1.47 95% CI: 1.05, 2.06). Respectively a 10 fold increase in PCBs levels was associated with increased risk (IRR 2.56 95% CI: 1.34, 4.91). Prenatal exposure to HCB was not associated with the risk of micronuclei frequencies. Clear monotonic exposure–response patterns were apparent for DDE and dioxin like PCBs.

Conclusion: These findings suggest that exposure to DDE and PCBs during pregnancy may increase the risk of micronuclei frequency in children. This is the first study to present prenatal POPs exposures and MN formation in later life and further studies are needed to confirm these findings and examine the biological mechanisms underlying the observed associations. Further follow up of this cohort will allow determining if prenatal exposure to POPs has, in addition, an effect on genetoxicity in late childhood and also long term carcinogenic risks.

Key words: persistent organic pollutants, genotoxicity, pregnancy, children, micronuclei

1. INTRODUCTION

Persistent organic pollutants (POPs) include chemicals such as polychlorinated biphenyls (PCBs) and the organochlorine pesticides hexachlorobenzene (HCB) and p,p-dichlorodiphenyldichloroethylene (DDE) (major metabolite of DDT) – a part of a group of chemicals considered as possible carcinogens and hypothesized to have genotoxic properties [1-4]. Because of their lipophilicity and long half-lives, they accumulate in the food chain, and the general population is still exposed to low doses of these substances [5-7] despite a ban (PCBs, HCB) or restriction (DDT) on the production and use since the 1970s by the Stockholm agreement [8].

Human exposure begins prenatally as many POPs can cross the placenta. After birth, exposure occurs through breast milk and also through inhalation (dust), ingestion (dairy and animal products), and skin contact [9].

It is considered that the potential pathways of exposure to organochlorines are mainly by oral through consumption of foods that contains small amounts of these compounds that subsequently cause bioaccumulation and bio-magnification [10, 11]. As a consequence, significantly high levels of these compounds can be observed in biological matrices such as adipose tissue, serum and human milk [12-14].

POPs possess endocrine-disrupting potentials. Endocrine-disrupting chemicals (EDCs) are compounds that either mimic or block endogenous hormones and thus disrupt the normal hormone homeostasis. The European Commission has defined EDCs as ‘an exogenous substance or mixture that alters the function(s) of the endocrine system, and consequently causes adverse effects in an intact organism or its progeny or subpopulation [15]. For example, some PCB congeners possess an oestrogenic potential (e.g. some hydroxy-PCBs), whereas others are anti-oestrogenic (e.g. PCB153, PCB180, PCB138) and anti-androgenic (PCB138); in addition, some have dioxin-like potentials (e.g. PCB126). Likewise, for OCPs, there has been reported both oestrogenic potentials [e.g. b-hexachlorocyclohexane (b-HCH), dichlorodiphenyltrichloroethane (DDT) and 1,1-Dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE)] and anti-androgenic effects (e.g. DDE) [16].

Exposure to persistent organic pollutants has been associated with a number of diseases, such as certain cancers, diabetes type 2 and cardiovascular disease [17]. Associations between

cancer and exposure to POPs have been investigated in more than 100 cohort and case-control studies from diverse countries as well as in many reviews of epidemiologic findings.

During the last two decades there's focus on in utero exposure of embryos to POPs and many cross sectional epidemiological studies and birth cohorts have been conducted in order to estimate exposures during pregnancy and establish epidemiological base data [18-25], also to investigate fetal exposure in accordance to mother's exposure [19-21, 26, 27] and to estimate the impact on the health of the offspring [28-36].

1.I. Persistent Organic Pollutants

ORGANOCHLORINES (DDT, DDE)

DDT is a chlorinated aromatic hydrocarbon insecticide that in its pure form exists at room temperature as colorless to off-white needles or powder with a slight aromatic odor. It is practically insoluble in water, but it is soluble in many organic solvents and highly soluble in lipids. It is very stable and exceptionally persistent in the environment. Technical-grade DDT is a mixture of three forms, p,p′-DDT (85%), o,p′-DDT (15%), and o,o′-DDT (trace amounts). Technical-grade DDT may also contain DDE and 1,1-dichloro-2,2-bis(p-chloro phenyl)ethane (DDD) as contaminants; both are breakdown products of DDT. DDE is the primary metabolite of DDT. From 1946 to 1972, DDT was one of the most widely used insecticides in the world. It was used for the control of insect pests such as the pink bollworm on cotton, codling moth on deciduous fruits, Colorado potato beetle, and European corn borer [4]. Trade names that DDT has been marketed under include Anofex, Cezarex, Chlorophenothane, Clofenotane, Dicophane, Dinocide, Gesarol, Guesapon, Guesarol, yron, Ixodex, Neocid, Neocidol and Zerdane [37]. In the public health field, DDT was used to control malaria, typhus, and other insect-transmitted diseases and to treat body lice. It was also used for mothproofing clothing. Its usage peaked in the 1960s, but in 1972, it was banned for the vast majority of uses in the United States. From 1946 to 1972, DDT was one of the most widely used insecticides in the world. It is also still used in many countries where malaria is endemic, as an insecticide to control mosquitoes.

The international agency for research on cancer (IARC) has categorized DDT as 2B which means that is possible carcinogen for humans [1] due to the fact that epidemiologic data are still insufficient [38].

Despite the 1972 U.S. ban of DDT, human exposure continues because of its extensive former use, its current use in some areas of the world, and the persistence of DDT and its breakdown products in the environment. DDT is still released into the atmosphere through spraying in some areas of the world. In addition, it volatilizes from soil in areas where it was formerly used. The volatilization and deposition cycle may be repeated many times, resulting in widespread distribution of DDT worldwide. In addition, DDT readily accumulates in animal fat and thus bio-accumulates through the food chain. DDT and its breakdown products have been found throughout the world, from the Arctic to the Antarctic, having been detected in ambient and indoor air, precipitation (rain and snow), water, soil, and animal and plant tissues. The residual levels of DDT in the environment have declined and continue to decline, but because of DDT's high persistence, it will be present at low levels for decades. In a study of long-term dietary intake of DDT and all of its metabolites, daily intake for a 70-kg 16-year-old U.S. male was estimated at 6500 ng for 1978–79, 2400 ng for 1979–80, 1500 ng for 1984–86, and 970 ng for 1986–91.

Currently, human exposure to DDT and its breakdown products is primarily through dietary ingestion, particularly of meat, fish, poultry, and root and leafy vegetables. The highest dietary exposure occurs among indigenous Arctic populations that eat traditional foods such as seal, whale, or caribou. The highest average daily intake was observed in the eastern Arctic, where total daily intake of DDT and all of its metabolites was 0.0242 to 0.0278 ng/day. The foods contributing the most were beluga whale blubber (0.316 ng/g of wet weight) and narwhal whale blubber (0.273 ng/g). DDT has been measured in numerous human tissues in the U.S. population and in other populations around the world, including indigenous Arctic peoples. DDT accumulates in fatty tissues and is usually found in higher concentrations in human milk than in cow's milk or other infant foods. In the United States, mean concentrations of DDT were 990 ng/g in milk fat from Arkansas women in 1986, 28.8 ppb (ng/g) in serum from consumers of Great Lakes fish in 1982, and 252 ng/g in adipose tissue from a national sample of individuals age 45 years or older in 1986. The median concentration of DDT in plasma samples from 407 highly exposed Inuit individuals living in Greenland was 0.035 ng/kg of lipid (35 ng/g). DDT was detected in 95% of the samples from this population. For the population measured in the United States National Health and Nutrition Examination Survey (NHANES), the geometric mean concentration of DDE in serum was 260 ng/g of lipid in 1999–2000, 285 ng/g in 2001–02 ,

and 238 ng/g in 2003–04. The Mexican-American population sampled in NHANES had mean DDE concentrations about twice those for the total population: 674 ng/g in 1999–2000, 652 ng/g in 2001–02, and 444 ng/g in 2003–04. Food and Drug Administration (FDA) Action levels for DDT in various food items and in processed animal feed range from 0.05 to 5 ppm [4]. The Maximum Residue Levels of DDT and its metabolites in the milk samples is 0.04 ng/kg for sum of DDTs as specified in EU Commission Regulation (EC) No 149/ 2008. In a study conducted in 2009-2010 in the Greek milk market in order to evaluate children's exposure to DDT and its metabolites via dietary milk consumption and to assess the respective potential risks to children's health in terms of cancer and non-cancer effects, 196 samples were collected and analysed. 191 (97.4%) were positive for DDT residues with mean value of 2.1 ng/ml [39]. In Greece exposure to POPs via maternal root has been reported twice. One time in the Rhea birth cohort in 2007 [40] and one time in breast milk in 2004 [41]. Results from the Rhea pregnancy cohort showed that in the first trimester of pregnancy DDT concentration in mother's blood, 0.0430 (0.041, 0.045) ng/ml and for DDE and 2.07 (1.97, 2.18) ng/ml [geometric mean (95% CI)] respectively, whereas in another study the p.p′-DDE concentration in breast milk was 530 ng/g lipidweight (lw) and p.p′-DDT 21 ng/g lipidweight (lw) (mean).

HCB

Hexachlorobenzene, or perchlorobenzene, is an organochloride with the molecular formula C_6Cl_6. It is a fungicide formerly used as a seed treatment, especially on wheat to control the fungal disease bunt. Previously, it was used as a seed-treatment fungicide for onions, sorghum, wheat, and other grains. Hexachlorobenzene was also used as a chemical intermediate in dye manufacturing, in the synthesis of other organic chemicals, and in the production of pyrotechnic compositions for the military. It was used as a raw material for synthetic rubber, as a plasticizer for polyvinyl chloride, as a porosity controller in the manufacture of electrodes, and as a wood preservative. In 2009, hexachlorobenzene was available from 19 suppliers worldwide, including 13 U.S. suppliers [4]. When hexachlorobenzene is released to the environment, it may be taken up by plants and animals and can bio-accumulate through the food chain. Hexachlorobenzene has been detected in terrestrial, freshwater, and marine food chains in the Great Lakes and Arctic regions. Populations with the greatest potential for exposure include those who ingest fish caught from contaminated water bodies or who reside near former

manufacturing or waste-disposal sites. According to EPA's Toxics Release Inventory, in 2008, 49 facilities released at total of 50.636 lb of hexachlorobenzene, mostly to on-site and off-site landfills. When hexachlorobenzene is released to air, it tends to remain mainly in the vapor phase and can therefore be transported great distances (for example, from temperate to polar regions). When released to water, hexachlorobenzene is strongly adsorbed to particles and sediment and is not degraded or hydrolyzed.

HCB has been banned globally under the Stockholm Convention on persistent organic pollutants [42]. It has also been categorized by the IARC to class 2B [2]. Hexachlorobenzene has been detected in the blood of numerous groups of people, especially indigenous populations of Arctic regions, in the blood and breast milk of pregnant and lactating women, and in the placenta and cord blood. In Arctic Canada, hexachlorobenzene was detected in all samples of maternal blood, and at higher concentrations in blood from Inuit women than from Caucasian women in the region. Cord blood plasma concentrations showed a similar trend. Breast-milk concentrations of hexachlorobenzene were elevated in populations of women who ate contaminated local fish in New York State and Finland. Hexachlorobenzene was found in all blood samples from pregnant women in an agricultural community in California. The diet of the Inuit population in Greenland was studied to determine the source of the high and increasing concentration of hexachlorobenzene. The blood levels of hexachlorobenzene in Greenland Arctic populations appeared to correlate with consumption of meals containing seal and whale. Hexachlorobenzene was detected in all adipose tissue samples collected at autopsy from Greenlanders. Hexachlorobenzene was detected in 98% of the blood samples collected from Akwesasne Mohawk youth living along the St. Lawrence River in New York State and Quebec; levels were somewhat higher in youths who had been breastfed as infants. In a study of consumers of sport fish in New York State, the mean blood hexachlorobenzene concentration was not significantly greater than that of non-consumers of sport fish [4]. The Food and Drug Administration (FDA) established the maximum permissible level in bottled water to be 0.001 mg/L [4]. In Greece in the Rhea birth cohort the detected concentration of HCB in mother's blood was 0.0894 (0.0865, 0.0925) ng/ml [geometric mean (95% CI)] [40], whereas in breast milk, HCBs median was detected 38 ng/g lw (mean) in the study of 2004-2005 in Thessaloniki [41].

PCBs

Polychlorinated biphenyls (PCBs) are a class of chlorinated aromatic hydrocarbons that consists of 209 congeners. They are highly stable and lipophilic chemicals widely distributed in the environment. Because of their general chemical inertness and heat stability, PCBs were predominantly used as coolants and lubricants in electrical equipment such as capacitors and transformers. Because of their non-flammability, chemical stability, high boiling point, and electrical insulating properties, PCBs were used in hundreds of industrial and commercial applications including electrical, heat transfer, and hydraulic equipment; as plasticizers in paints, plastics, and rubber products, in pigments, dyes, and carbonless copy paper; and many other industrial applications.

IARC has categorized PCB-126 as carcinogen for humans (category 1) [3].

They were produced commercially from 1929 to 1970s as their high dielectric properties made them so useful. PCBs were manufactured as a mixture of PCB congeners under a variety of names, the most common being Aroclor. Aroclor mixtures of chlorinated biphenyl congeners vary in degree of chlorination. Once commercial PCB mixtures are released into the environment, processes such as volatilization, partitioning, chemical or biological transformation, and preferential bioaccumulation alter them and dictate their environmental fate. These processes are dependent on the degree of chlorination of the biphenyl molecule. The higher chlorinated PCB congeners adsorb strongly to sediment and soil, where they tend to persist with half-lives from months to years. PCBs also bio-accumulate in the food chain, preferentially in fatty tissues [7].

1.II.Micronuclei

MN are small extra-nuclear bodies that result from acentric chromosome/chromatid fragments or whole chromosomes/chromatids that lag behind in anaphase stage and are not included in the daughter nuclei in telophase stage [43]. Instead, they are enwrapped by the nuclear membrane and resemble the structure of the daughter nucleus, although being way smaller in size [44, 45]. Whole chromatids or chromosomes in MN are formed due to deficiencies in chromosome segregation during anaphase usually caused by mitotic spindle failure, kinetochore damage, centromeric DNA hypomethylation, and defects in the cell cycle control system [46]. According to Fenech et al. (2011), the main mechanism of MN formation

originated from chromosome malsegregation is hypomethylation of centromeric and paracentromeric regions – satellite repeats. Usually, satellites are hypermethylated, and loss of methylation elongates repeat regions decreasing the tension in kinetochores and thus creating wrong connections between microtubules of the mitotic spindle and chromosomes [44]. Sometimes, chromatids/chromosomes are unable to segregate as the mitotic spindle cannot pull them apart due to tubulin de-polymerization. The absence of kinetochore or centromeric defects also lags chromosomes behind at telophase [47].

MN indicate genome damage and MN frequency in peripheral blood lymphocytes has been associated with increased risk of cancer in adults [48-50]. The contribution of epigenetic alterations to MN formation is now clearly evident, and research on the epigenetic mechanisms involved in MN is growing [47]. Recently, the combination of next-generation DNA sequencing, single nucleotide polymorphism array analyses and bioinformatics methods has revealed a striking new phenomenon, termed chromothripsis (from the Greek for 'chromosome' (chromo) and 'shattering into pieces' (thripsis)), in which one or a few chromosomes in a cancer cell bear dozens to hundreds of clustered rearrangements [51]. A recent study by Zhang et al. [52] has now revealed a molecular mechanism that causes chromothripsis. The work of Zhang et al. demonstrates that DNA damage of lagging chromosomes within the context of a micronucleus is one mechanism causing chromothripsis. The paper confirms that both random joining of shattered chromosome fragments and template-switching resulting from fork stalling during DNA replication contribute to chromothripsis [53, 54]. Chromothripsis, may affect cancer gene function and thereby have a major impact on cancer progression, prognosis, and therapy response [55, 56].

Nonetheless the formation of micronuclei is considered to be an effective biomarker of diseases and processes associated with DNA damage. The cytokinesis-block micronucleus (CBMN) assay is one of the standard cytogenetic tools employed to assess chromosomal damage subsequent to exposure to genotoxic/cytotoxic agents, and is widely applicable to plant, animal and human cells [57]. Micronuclei have been used to various occasions as a biomarker for age, sex, and exposure to radiation, chemicals, and mother's nutrition during pregnancy [58-65]. Moreover the biomarker has detected DNA damage in adults [66, 67] and children [25] exposed to POPs. It was also used to detect a net of marker genes in children exposed to atmospheric pollution [63, 68], genetic damage of children exposed to organophosphates [69], to explore

genotoxicity in newborns [70, 71] and to identify genes and genetic polymorphisms is newborns that relate to children's leukemia [72, 73].

The efficacy of the biomarker to detect the effect of p,p'-DDT on human genome has been proved also in vivo studies with micronuclei scoring according to the criteria proposed by the HUMN project [74, 75].

1.III. Genotoxic effects humans

DDT/DDE

Associations between DDT and non-Hodgkin's lymphoma (NHL) has been reported in two case control studies since the eighties [76, 77] and many more followed and were reviewed by Pierce in 2005 [78]. Epidemiological data remain inconclusive as some studies fail to find a significant association between DDT and NHL whereas many others succeed it. No association has been demonstrated by the review of three case control studies from Baris et al in 1998, the Epilymph Study, a nested case control study in the U.S. in 1997, a case control study among three European countries in 2008, a Swedish case control study, and a population-based case-control study in the United States by De Roos et al in 2005 [79-84]. On the contrary 11 studies including the Agricultural Health Study in the U.S. suggested an increase in non-Hodgkin's Lymphoma due to DDT or DDE [85-95]. Up to now, we haven't recovered a systematic review or a meta-analysis, that follows the suggestions of PRISMA [96], regarding NHL and organochorines. DDT has been associated with leukemia [81, 87, 90] and prostate cancer [97-101].

Two meta-analyses of the epidemiologic evidence on DDT burden and breast cancer risk have been performed in 2004 and 2013. In the first no association has been detected whereas in the latter also a small non-significant association has been demonstrated [DDE (1.05;95% CI: 0.93–1.18) and DDT (1.02; 95% CI: 0.92–1.13)] [102, 103]. The association has been further explored in nested case control study among the cohort population of the Child Health and Development Studies. After 54 years of observation in 9300 daughters that participated in the cohort, 118 women developed breast cancer until the age of 52. This rate after matching to the controls demonstrated that mother's exposure to DDT had a 3.7 fold higher risk of daughter's breast cancer to non-exposed mothers to DDT [104]. From the same cohort a previous nested

case control study had also shown that women exposed to DDT under the age of 14 had presented a five- fold higher breast cancer risk than non-exposed women [104, 105]. In a recent case control study in Tunisia a high association was presented on the exposure to organochorines (DDE) and breast cancer which did not remain statistically significant after including DDT and HCB to the multivariate analysis [106].

Nested and population based case-control studies in China reported strong, dose-related associations between liver cancer and blood DDT level after adjustment for potential confounders [107-109]. No excess risk of liver cancer was reported in a historical cohort study of men who sprayed DDT during a malaria control campaign in Italy [38, 110]. A case-control study using a geographic information system (GIS) to link SEER-Medicare and California pesticide data linked organochlorine exposure and the risk of hepatocellular carcinoma [111]. Several case-control studies in the USA and Europe reported positive associations between DDT or DDE and testicular cancer, including a large case-control study nested in a US military cohort [38, 112]. No excess risk was found between DDT and childhood leukemia in the review and meta-analysis of the epidemiologic evidence in 2009 [113].

There is strong evidence that DDT affects several mechanisms that can operate in humans. Immunosuppression has been consistently observed in numerous experimental systems, including human cells in vitro [114-117]. DDT, DDD, and DDE increased oxidative stress in human peripheral blood mononuclear cells [118, 119] and stimulated human colon cancer [120] and liver cancer cell proliferation in vitro [121, 122]. Estrogenic effects and androgen-receptor antagonism were consistently observed in numerous experimental systems including human cells in vitro [123]. Anti-oestrogens blocked oestrogenic effects of DDT in human breast cancer cells [124].

Epigenetic mechanisms such as DNA methylation, histone modifications and microRNA expression are heritable changes in gene expression that occur without change in the DNA sequence. DNA methylation is important in normal processes of development and genomic imprinting. The interest for epigenetic changes have grown in recent years, since it has been shown that epigenetic changes are associated with cancer [125]. DNA hypomethylation was seen in breast cancer cells [17]. Ruiz-Hernandez et al recently investigated the bibliography of endocrine disruptors on DNA methylation [9]. There were four epidemiologic studies investigating the association between POPs [17, 126-128], respectively with DNA methylation in

adults. These studies were conducted in South Korea [129], Sweden [17], Denmark [128], and Japan [126]. In studies assessing POPs, exposure was measured in plasma [128] or serum [17, 126]. For most POPs, studies evaluating DNA methylation globally showed a trend towards hypomethylation with increasing levels of exposure [126, 128, 129]. The two studies measuring DNA methylation in Alu elements [128, 129] showed consistent statistically significant inverse associations with p,p′-DDE and DDT. In a population of Japanese women (N= 399), serum POPs were inversely associated with the global DNA methylation level measured by LUMA [126, 129]. In an elder population from Sweden (N= 519) [17], however, increasing p,p′-DDE concentrations was significantly associated with increasing global DNA methylation levels measured by LUMA ($P < 0.05$) [17].

In children levels of DNA methylation were lower in 9-year olds compared to newborns and were higher in boys compared to girls. Higher prenatal DDT/E exposure was associated with lower Alu methylation at birth, particularly after adjusting for cell type composition ($p=0.02$ for o,p′ -DDT). Associations of POPs with LINE-1 methylation were only identified after examining the co-exposure of DDT/E with PBDEs simultaneously. These data suggest that repeat element methylation can be an informative marker of epigenetic differences by age and sex and that prenatal exposure to POPs may be linked to hypomethylation in fetal blood [130].

HCB

A case control study has shown an association between endometrial exposure to HCB and testicular cancer to the progeny [131]. We did not find any study that suggests an association of HCB to childhood cancer.

HCB activates c-Src/HER1/STAT5b and HER1/ERK1/2 signaling pathways and cell migration, in an AhR-dependent manner in MDA-MB-231 breast cancer cells [132].

PCBs

A meta-analysis of epidemiologic data has shown that PCBs contribute to non Hodgkin's lymphoma (OR 1.5, 95%CI: 1.1-1.7) [84, 133]. Prostate cancer risk due to exposure has been suggested by case control studies [134-137] though two later reviews haven't confirmed these findings [133, 138]. Women who have been found to be exposed to PCBs right after giving birth presented an elevated risk of developing breast cancer according to a nested case control study

among the among the population of the Child Health and Development Studies [139]. Likewise prostate cancer, later reviews of the epidemiological data also did not suggest an association between PCBs and breast cancer [106, 133, 138], even though there has been made much progress on the epigenetic mechanisms of these substances [140, 141]. PCBs have been associated with testicular cancer in a case control study [142] and also higher concentrations from in utero exposure has been proved to testicular cancer patients [131]. Exposure from working environment has not been associated with testicular cancer of the progeny [143], thought children's exposure to PCBs through PCB 118, 138, and 153 in household dust has been associated with 2 fold risk of leukemia in the findings of a meta-analysis by Ward et al in 2009 [(OR) = 1.97; 95% confidence interval (CI), 1.22–3.17] [113].

Overall, all PCBs can induce formation of reactive oxygen species, genotoxic effects, immune suppression, an inflammatory response, and endocrine effects to various extents and through different pathways. The dioxin-like PCBs exert their effects mainly through AhR activation and the downstream cascade of related events; less-chlorinated PCBs act more readily through metabolic activation and the downstream effects of these metabolites. Thus, mixtures might have more than additive effects. [144] Like TCDD, 2,3,4,7,8-pentachlorodibenzofuran and 3,3',4,4',5-pentachlorobiphenyl (PCB-126) are complete carcinogens in experimental animals,7,8 and there is extensive evidence that they act through the same AhR-mediated mechanism. [145]

Increasing PCB183 and PBDE47 in a study population from Korea (N= 86) and PCB 156, 99, and 105, sum of PCBs, and sum of POPs in a study population from Denmark (N= 70) was significantly associated with lower DNA methylation in Alu elements [128, 129]. Consistently, in a population of Japanese women (N= 399), serum POPs were inversely associated with the global DNA methylation level measured by LUMA [126]. In an elder population from Sweden (N= 519) [17], however, increasing PCB126 concentrations was significantly associated with increasing global DNA methylation levels also measured by LUMA ($P < 0.05$) [99].

The enhanced metastatic potential of polychlorinated biphenyls (PCBs) in breast cancer cells was linked to activation of Rho-associated kinase [146]. Both animal experiments and epidemiological data suggest that fetal exposure to endocrine-disrupting compounds (a class of environmental toxicants that interfere with the endocrine signaling pathway that include BPA,

phthalate, pesticides, PCBs, dioxins, etc.) increase the incidence of adult breast and PCas in the general population [147, 148].

Testing whether exposure to chemical could disrupt endothelial integrity and increase the transendothelial migration of tumor cells, investigators exposed human microvascular endothelial cell 1 to PCB 104, a representative of highly orthosubstituted non-coplanar PCB congeners, and tested their effect on the endothelial permeability and transendothelial migration of MDA-MB-231 breast cancer cells. They reported that PCB 104 induced endothelial hyperpermeability and markedly increased transendothelial migration of MDA-MB-231 cells. These effects were associated with overexpression of vascular endothelial growth factor through PI3K, but independent of NF-κB pathways [149]. In a follow-up study, this team also reported that PCB exposure of endothelial cells stimulated transendothelial migration of tumor cells through upregulation of MMP3. The study provided evidence that PCB can activate EGFR and JAK3 in a closely coordinated and cross-dependent fashion. Activated EGFR and JAK3 stimulate in concert kinases c-JNK and ERK1/2 as well as increase DNA-binding activity of AP-1 and polyoma virus enhancer activator protein 3, leading to transcriptional upregulation of MMP3 expression [150].

PCB quinone is cytotoxic in HepG2 cells [151]. PCB29-pQ exposure significantly increased olive tail moment (OTM) and micronuclei (MN) frequencies in HepG2 cells. These data suggested that PCB29-pQ caused DNA strand breaks and chromosome breaks. The level of oxidative DNA damage was significantly evaluated with PCB29-pQ exposure concentration and time dependently. Moreover, c-H2AX appeared after the treatment of PCB29-pQ in HepG2 cells, may indicate double strand breaks (DSBs). In addition, the pretreatment of ROS scavengers inhibited the genotoxicity of PCB29-pQ significantly. PCB29-pQ causes genotoxic effects in HepG2 cells, probably via ROS-induced oxidative DNA damage [152].

1.IV. Genotoxic effect animals

DDT/DDE

DDT caused liver tumors in two rodent species and by two different routes of exposure. It caused primarily malignant primary liver-cell tumors (hepatocellular carcinoma) in mice of both sexes and in rats (of unspecified sex) following dietary exposure; in mice of both sexes following

administration by stomach tube; and in female mice following subcutaneous injection [4, 153]. Increased incidences of lung tumors and malignant lymphoma following oral exposure to DDT were observed in some, but not all, of the studies in mice. However, there is evidence for a dose threshold and exceedingly low doses may even act in a hormetic fashion, inhibiting development of the lesions as described later [154]. Effects of long-term oral administration of DDT on nonhuman primates were evaluated on 24 cynomolgus and rhesus monkeys. In 1969 DDT (20 mg/kg) was given in the diet for 130 months, followed by an observation period that ended in 1994 [155]. The results of the study provided evidence of carcinogenicity in non-human primates confirming the carcinogenicity confirmed in rodents [156].

Hormesis has been defined as a dose–response relationship in which there is a biological activation at low doses but an inhibition at high doses, or vice versa, resulting in a U, J or inverted U-shaped dose–response. Hormetic effects have been studied for more than two decades and many toxicants have shown benefits, rather than harm, with low-level exposure [157]. Inhibitory effects on the induction of GST-P positive foci were also noted with low doses of DDT [158]. First, in the study of Sukata et al. [158], F344 rats, 21-day-old at the commencement, were administered DDT at doses from 0.005 to 500 p.p.m. in their diet for 16 weeks. In another experiment Kushida et al. [159] investigated the possibility of hormesis after DDT administration to F344 rats for 11 and 43 weeks following initiation of hepatocarcinogenesis using DEN. In both experiments the doses of 20 p.p.m. were associated with dose-dependent induction of GST-P positive foci in the liver. In contrast, 0.005 and 0.01 p.p.m. administration resulted in a tendency for decrease in values below the control level. Histopathological analysis of liver nodules also revealed a tendency for decrease in the incidence and multiplicity of HCCs in the low-dose groups as compared with the DEN initiation controls. The multiplicity of total tumors also tended to decrease, although incidences were similar. Alteration of the GST-P positive foci in the low-dose groups was correlated with a tendency for decrease in the CYP3A2 protein level as well as induction of IL-1 receptor type I (IL-IRI) and TNF-a receptor type I, whose ligands have roles in downregulating CYP3A2 and influencing cellular proliferation or apoptosis [158]. IL-1R1 is known to be a cell surface molecule involved in cell signaling [160], while IL-1 inhibits regeneration of rat liver cells [161] and tumor cell growth [162] and inhibitory actions of IL-1b on hepatocyte DNA synthesis are effected by iNOS gene expression and NO production under IL-1R1 control [163]. It was found that within GST-P

positive areas, cell proliferation was slightly lower in the 0.005 p.p.m. DDT dose group than in the only DEN treated group [158]. As observed in experiments with phenobarbital and a-BHC, CYP2B1/2 and CYP3A2 protein levels in the liver microsomal fraction were significantly elevated by high doses of DDT. In line with previous results, 8-OHdG formation was significantly suppressed by a low dose of the chemical, presumably related to effective DNA repair and co-repair of endogenous damage, which may exceed formation of adducts [164]. Oxidative stress in the low-dose group was suggested to be decreased because of the lowered CYP3A2 expression and formation of 8-OHdG balanced through elimination by Ogg1 [158, 159]. Furthermore, in the low DDT dose group, mRNA expression and immunohistochemical staining of connexin 32 (Cx32) were found to be elevated [158]. Many previous studies indicated that high doses of DDT and other non-genotoxic carcinogens inhibit Cx32, resulting in the loss of the function of gap junction intracellular communication (GJIC) [159-169] and release of potentially initiated cells from growth constraints imposed by normal neighboring cells, resulting in clonal expansion and ultimately tumor formation and progression [169, 170]. In the study of Fukushima et al in 2005 [157], mRNA expression of one of the transcriptional factors, HNF-1a, which regulates Cx32 expression [171, 172] was in good correlation with that of Cx32 [165]. Differential alteration of HNF-1a is suggested to be one of the possible mechanisms by which DDT might inhibit or promote rat hepatocarcinogenesis.

Another theory of hepatocarcinogenesis is that DDT treatment may result in cell cycle progression and apoptosis inhibition through constitutive androstane receptor CAR- and estrogen receptor ERα-mediated gene activation in mouse livers. Findings suggest that the proliferative and anti-apoptotic conditions induced by CAR and ERα activation may be important contributors to the early stages of hepatocarcinogenesis as produced by DDT in rodent livers [173]. The regulation of PXR/CAR/ER-target genes in the mouse and rat liver is elicited by o, p'-DDT in a species-specific manner of regulation of PXR/CAR/ER-target genes. DDT is known to cause liver tumors in both mice and rats, marked species differences in PXR/CAR structure, expression patterns and ligand preference as well as significant species-specific differences in steroidogenesis, especially CYP17A1 expression and activity [174, 175].

In addition to PXR-, CAR- or ER-mediated gene expression changes, o, p'-DDT induced Gadd45a, Gadd45b and Cdkn1, all of which are DNA damage-responsive genes [176, 177]. Consistent with this are the reports of the DNA damaging potential of DDT [178, 179].

Consequently, DNA damage may be an additional risk factor for tumor initiation/promotion following o, p'-DDT exposure in addition to PXR/ CAR- and ER-mediated activities. Considering that the induction of DNA damage-responsive genes precedes Cyp2b10 or Cyp3a11 induction, the DNA damage may not be caused by oxidative stress derived from enzyme induction. Moreover, Gclm and Hmox, both known oxidative stress-responsive genes [180, 181] exhibited relatively weak induction compared to rats [175], suggesting that oxidative stress was not strongly induced. This includes PXR/CAR- and ER-mediated responses, altered steroidogenesis, oxidative stress, and DNA damage. Although DDT is known to cause liver tumors in both mice and rats, the marked species differences in PXR/CAR structure, expression patterns and ligand preference as well as significant species-specific differences in steroidogenesis, especially CYP17A1 expression and activity, confound the extrapolation of these results to humans [174].

The evaluation of the genotoxic effect of DDT exposure in oral mucosa cells, lymphocytes and mammary gland epithelial cells in adult female rats showed a significant increment in the lipid peroxidation rate in mammary cell membranes in DDT exposed rats in comparison to animals from both control groups. Adult rats chronically exposed to DDT showed high a lipid peroxidation rate in their mammary tissue, reflecting an oxidative stress condition [182]. It is a well-known fact that oxidative stress plays a very important role in the carcinogenesis process; also, some facts indicate that reactive oxygen species are involved in cancer early stages and in its progression [183, 184].

There are some studies evaluating the effect of POPs and other endocrine disruptors on DNA methylation in experimental settings. Exposure to DDT induced hypomethylation of CpG islands in Sst, Gal, Arf1, Ttr, Msx1,and Grifin genes in the hypothalamus of young male rats. Rats treated in utero and postnatally with organochlorine pesticides and PCBs also showed decreased methylation of CpG sites in the promoter of the tumor suppressor gene p16 (INK4a) compared to controls [9].

HCB

HCB did not induce chromosomal aberrations in cultured Chinese hamster cells [185] and dominant lethal mutations in rats [186, 187], but it has been found to produce liver tumors in mice [188-193], rats [190, 192, 194-196] and hamsters [189, 190], as well as an increased

incidence of renal-cell adenomas, parathyroid adenomas and adrenal pheochromocytomas in rats [197] and of thyroid adenomas in hamster [189]. Canonero, R. et all in 1990 noticed that HCB did not induce DNA fragmentation but produced an increase in the frequency of micronuclei in rat hepatocytes. The formation of micronuclei in the absence of DNA damage should be interpreted as due to an aneugenic activity, rather than to the occurrence of a clastogenic effect and assumed that HCB is transformed in the liver and acts as a week genotoxic carcinogen [198]. This hypothesis is supported by its ability to induce tumors in more than one species and multiple target sites, a typical property of mutagenic carcinogens [199].

In vitro the effect of HCB (0.005, 0.05, 0.5, 5muM) on cell invasion and metalloproteases (MMPs) 2 and 9 activation in MDA-MB-231 cells showed that HCB (5muM) enhances MMP2 expression, as well as cell invasion, through AhR, c-Src/HER1 pathway and MMPs. Moreover in vivo the effect of HCB (0.3, 3, 30mg/kg b.w.) on tumor growth, MMP2 and MMP9 expression, and metastasis using MDA-MB-231 xenografts showed that HCB increases MMP9 expression, secretion and activity through a HER1 and AhR-dependent mechanism, in MDA-MB-231 cells. HCB (0.3 and 3mg/kg b.w.) enhances subcutaneous tumor growth in MDA-MB-231 and C4-HI in vivo models. In vivo, using MDA-MB-231 model, the pesticide (0.3, 3 and 30mg/kg b.w.) activated c-Src, HER1, STAT5b, and ERK1/2 signaling pathways and increased MMP2 and MMP9 protein levels. Furthermore, HCB stimulated lung metastasis regardless the tumor hormone-receptor status. These findings suggest that HCB may be a risk factor for human breast cancer progression [132].

HCB is a ‘‘dioxin-type’’ chemical and a weak agonist of the aryl hydrocarbon receptor (Ahr) [200], a ligand-activated transcription factor. It has been proposed that the binding of ‘‘dioxin-type’’ chemicals to the Ahr complex could activate the phosphorylation of important proteins in the cytosol, plasma membrane, and other intracellular organelles, eliciting changes in signal transduction and gene expression activities [201]. In vivo exposure to HCB caused profound changes in some biochemical parameters of rat liver membranes such as microsomal protein phosphorylation, down-regulation of Epidermal Growth Factor Receptors (EGFR) and a dose-dependent increase in EGFR-tyrosine kinase activity [202, 203]. Insulin, insulin-growth factors (IGFs), and their corresponding receptors (IR, IGF-IR, and IGF-IIR) participate in the growth and proliferation of normal and neoplasticmammary cells [204]. The IR is a potential oncogene for mammary epithelial cells since its content is in-creased in most human breast

cancer biopsies, and both ligand-dependent malignant transformation and ligand-dependent enhanced growth occurs in cultured breast cells overexpressing the IR [205]. Insulin receptor substrate-1 (IRS-1) was originally identified downstream of the IR but can also be phosphorylated in response to IGF-IR a3ctivation [206]. Following activation, IRS-1 binds a diverse set of downstream signaling molecules including p85, Grb2, Nck/Crk, Syp/Fyn, and SHP2 [207]. The complexity of IRS-1 upstream and downstream signaling reflects an emerging concept of complex cross-talk between extracellular and intracellular signaling pathways and may place IRS molecules in a central position to coordinate multiple signaling pathways [208]. Because IR, IGF-IR, and IRS-1 play an important role in both cell proliferation and cell transformation, Randi et al in 2006 explored mechanism of action of HCB in mammary tumor induction in rats by N-nitroso-N-methylurea (NMU). The results showed that HCB altered insulin/IGF-I signaling pathway, at least with regard to IR and IGFR-I expression as well as IRS-1 content and tyrosine phosphorylation. These observations suggest that the IGF-IR signaling pathway may be involved in HCB tumor co-carcinogenic action [209].

The combined effect of organochlorine pesticides heptachlor (HEP) and hexachlorobenzene (HCB) was evaluated by using a medium-term rat liver bioassay. Male F344 rats were initially administered diethylnitrosamine (DEN, 200 mg/kg i.p.); after a 2-week non-dosing period, they were given diets containing HEP (5 or 25 ppm), HCB (70 or 350 ppm), or their mixtures (5 and 70 ppm or 25 and 350 ppm) for 6 weeks. All rats were subjected to partial hepatectomy at week 3 and killed at week 8. Additive or synergistic effects of HEP and HCB were observed in groups treated with mixtures of these pesticides. Number and area of preneoplastic foci positive for glutathione S-transferase placental form (GST-P) were consistently higher in these groups than the sum of individual values in the groups treated with HEP or HCB alone. Consistent with these findings, HEP and HCB had additive or synergistic effects on cell proliferation induction within the preneoplastic foci and cytochrome P450 (CYP) 2B1 and 3A1 induction, which may lead to more efficient metabolic activation of HEP and HCB. HEP and HCB have additive and synergistic effects on the development of GST-P-positive foci and that higher risks are associated with a combination of residual organochlorine pesticides in foods than with individual residual organochlorine pesticides [210].

PCBs

PCBs, complete carcinogens and promoters in the rodent liver [211], affect both cellular proliferation and apoptosis [212]. They may act as promoters in the mouse lung [213] and may be associated with accumulation of iron in Kupffer cells and elevated proliferation of hepatocytes in the rat liver [214]. There is evidence that neonatal exposure to high doses of organochlorines could favor the development of MNU-induced mammary lesions, but it is also reported to delay the development of palpable tumors in the rat [215-217]. PCB 118 in female Harlan Sprague-Dawley rats increased incidences of neoplasms of the liver (cholangiocarcinoma, hepatocholangioma, and hepatocellular adenoma) and cystic keratinizing epithelioma of the lung. Occurrences of carcinoma in the uterus were considered to be related to the administration of PCB 118. Occurrences of squamous cell carcinoma of the uterus and acinar neoplasms of the pancreas may have been related to administration of PCB 118. Administration of PCB 118 caused increased incidences of nonneoplastic lesions in the liver, lung, adrenal cortex, pancreas, thyroid gland, nose, and kidney [218]. Exposure of rats to mixture of TCDD, PCB126, and PeCDF induced chronic active inflammation in the ovary (PCB153, binary mixture of PCB126 and PCB153), acute and/or chronic active inflammation of the uterus (PeCDF, PCB153), cystic endomtrial hyperplasia binary mixture of PCB126 and PCB153), and uterine carcinoma (PeCDF, PCB118). These effects were possibly via anti-estrogenic mechanisms, endocrine disruption of the reproductive organs, or a local retinoid deficiency pathway resulting in abnormal circumstances or epithelial differentiation [219].

In xenograft mouse model, PCBs significantly advanced disease progression, leading to enhanced capability of metastatic breast cancer cells to metastasize to bone, lung and liver [146].

These congeners and mixtures include AhR agonists that exhibit dioxin-like activities, and CAR agonists. Other PCB congeners (PCB153) and low-chlorinated commercial products, which were less well studied than highly chlorinated products, showed limited evidence of carcinogenicity in experimental animals. The relative contributions of different PCB congeners to the carcinogenicity of the commercial mixtures are not known. Overall, all PCBs can induce formation of reactive oxygen species, genotoxic effects, immune suppression, an inflammatory response, and endocrine effects to various extents and through different pathways. The dioxin-like PCBs exert their effects mainly through AhR activation and the downstream cascade of related events; less-chlorinated PCBs act more readily through metabolic activation and the

downstream effects of these metabolites. Thus, mixtures might have more than additive effects [144].

1.V. Micronuclei formation related to POP exposure

Investigations of acquired chromosomal changes have focused on their association with particular types of neoplasia. The gold standard for scoring acquired chromosomal abnormalities has been the evaluation of metaphase chromosomes. Although this technique allows for the characterization of all cytogenetic findings present, it is limited because it's labor intensive and presets a risk for producing 'artifactual' anomalies. An alternative approach that has been used to estimate the frequency of acquired chromosomal changes is the cytokinesis-block micronucleus (CBMN) assay, which provides information regarding the presence of chromosomal errors in somatic cells prior to the influence of selective growth pressures [220]. Given that this methodology allows for an assessment of a large number of cells (1000 or more) and is less labour intensive than conventional cytogenetic studies, it has potential for use as a high-throughput assay. Micronuclei, which are the primary cytological structures scored in the CBMN assay, are thought to contain chromatin (from one or more chromosomes) that was not incorporated ('lagging' or 'lost') into the daughter binucleates following nuclear division [220]. Micronuclei frequencies have been shown to increase with both age and DNA damage, providing data that closely parallels that of metaphase chromosomal analyses [221, 222]. Thus, the assessment of micronuclei frequencies has become a very attractive bio-surveillance tool for quantifying genomic damage associated with environmental insults and occupational exposures, as well dietary and lifestyle practices [223]. As anticipated from the above noted observations regarding chromosomal changes and age-related conditions, researchers have also reported micronuclei frequencies to be increased in individuals with different health problems, especially age-related conditions such as cancer. All the above are described in a twin study which also indicated that influences from both additive genetic (65.2% of variance) and unique environmental (34.8% of variance) sources explain the observed micronuclei frequencies in monozygotic and dizygotic twin pairs [224].

The Human Micronucleus (HUMN) project (www.humn.org), an international collaborative project aimed at determining the variables affecting MN frequency in humans and the pathological significance of this biomarker, has completed a cohort study involving a total of

6,718 subjects from ten countries, screened for MN frequency between 1980 and 2002; a significant increase of all cancer incidence was found for subjects in the groups with medium (RR ¼ 1.84; 95% CI: 1.28–2.66) and high MN frequency (RR ¼ 1.53; 95% CI: 1.04–2.25) relative to those with low MN frequency [48, 57, 225].

Cancer and childhood cancer have been associated with exposure to pesticides, organic persistent pollutants and agriculture as described earlier and children are particularly vulnerable to them due to their unique age-related behaviors [226-228]. Their route and source of exposure differ from those observed in adults [25, 229]. Therefore, children are at high risk of additional health problems from environmental exposure to pesticides [230, 231]. The exposition of the adverse health effects of these exposures in utero, in the early stages of life has been studied and such hypotheses have been made [69, 104, 131, 148, 232-235].

Little information is available about micronuclei frequencies in children and prenatal exposures to persistent organic pollutants. We found only two studies on the association of mother's exposure and exposure to persistent organic pollutants at birth with micronuclei frequencies as an outcome in childhood [236, 237]. Alvarado-Hernandez et al, 2013 determined hexachlorobenzene, p'p'-DDT and p'p'-DDE and micronuclei frequencies in mother–infant pairs. The micronuclei frequencies were higher in mothers than in umbilical cords (UC) and the levels of micronuclei were not correlated with pesticide levels of the mother's nor the UC samples [236, 237]. In the BioMadrid study father-pregnant woman-newborn trios were investigated for the association between micronucleus frequency and PCBs and results have not been presented yet [236, 237]. In our bibliographic research we did not find such an association in a prospective study

1.VI. Hypothesis- target

Much theoretical evidence has been accumulated supporting the causal role of MN induction in cancer development and prospective cohort studies are needed to validate MN as a cancer risk biomarker [49]. Our aim was to investigate for the first time the association between in utero exposure to POPs with MN frequency in lymphocytes of preschool children from the Rhea mother-child cohort in Crete, Greece.

2. MATERIALS AND METHODS

2.I. Study population

This study is a part of the 'Rhea' mother and child-cohort study. The Rhea study prospectively examines a population-based sample of pregnant women and their children at the prefecture of Heraklion, Crete, Greece. Methods are described in detail elsewhere [238]. Briefly, female residents (Greek and immigrants) who became pregnant during a period of one year starting in February 2007 were contacted and asked to participate in the study. The first contact was made at the time of the first comprehensive ultrasound examination (mean ± SD 11.96 ± 1.49 weeks) and several contacts followed (6th month of pregnancy, at birth, 9 months, 1st year, 4 years and 6 years after birth). To be eligible for inclusion in the study, women had to have a good understanding of the Greek language and be older than 16 years of age. Face-to-face structured questionnaires along with self-administered questionnaires and medical records were used to obtain information on several psychosocial, dietary, and environmental exposures during pregnancy and early childhood. The study was approved by the ethics committee of the University Hospital in Heraklion, Crete, Greece, and all participants provided written informed consent after complete description of the study.

Of 1363 singleton live births in the Rhea study, POP concentrations in maternal serum samples were measured for 1135 subjects and MN frequencies in 1000 binucleated T-lymphocytes were measured in a random sample of 369 children at 4 years of age.

2.II. Exposure

Sample collection

Maternal serum samples were collected at the first prenatal visit around the 3rd and 4th month of pregnancy, in 10 ml Silicone gel separator vacutainer (Becton Dickinson, UK), were centrifuged within 2 hours from blood collection at 2500rpm for 10min and were then stored in aliquots at - 80°C until assayed.

POP analysis

The POP analyses were performed in the National Institute for Health and Welfare, Chemical Exposure Unit, Kuopio, Finland with an Agilent 7000B gas chromatograph triple

quadrupole mass spectrometer (GC-MS/MS). Pretreatment of serum samples for GC-MS/MS analysis has been described elsewhere [239]. Serum concentrations of six individual PCB congeners (IUPAC numbers: 118, 138, 153, 156, 170 and 180), HCB, DDT and DDE, and BDE-47 were determined. All the results were reported on whole weight and expressed in pg/ml serum, while samples bellow the limit of quantification (LOQ) were assigned the value 0.5×LO Q. LOQ was 6 pg/ml for PCB118 and PCB 156; 10 pg/ml for HCB, DDE, PCB138, PCB153, PCB170, PCB180 and BDE47, and 50 pg/ml for DDT. We chose to use wet-weight levels for the POPs but adjusted for fasting maternal serum triglycerides [mean (±SD) = 130.3 (58.9) mg/dl] and cholesterol [212.7 (43.1) mg/dl] as continuous variables in all multivariable models to minimize potential biases associated with automatic lipid adjustment [240]. The percentage of samples with levels of DDT above the LOQ was 34.1%. For PCB 156 and BDE-47, 55.6% and 22.3% were above the LOQ, respectively. Due to high percentages of samples below the LOQ, DDT and BDE-47 were not used in the statistical analyses. POPs were treated as continuous variables on a $\log_{10}$ scale. We calculated total PCB concentrations by summing the concentrations of the 6 individual PCB congeners and estimated associations using separate models for DDE, HCB, and the sum of PCBs.

2.III. Outcome

Sample collection

The protocol for blood sampling that was followed at the PAGNI hospital involved precautions in order not to cause discomfort to the children. As so 30 minutes prior to the blood draw, the anesthetic cream EMLA that consists of lidocaine and xylocaine was applied and covered with a bandage. 10 ml of peripheral lymphatic blood was collected from each child in heparinized tubes (BD Vacutainer, Plymouth, UK). The tubes were transferred within two hours to the Laboratory of Clinical Nutrition and Epidemiology in the Department of Social Medicine of the Medical School of Crete where the preparation of the biological sample for the micronuclei enumeration took place.

Sample preparation

For the preparation of the cells we used the protocol that has been developed for the European Project New Generis [241]. According to the protocol 0.4 ml of whole blood was

cultured to RPMI (Gibco, Germany) media with phytohemaglutatinin A 16 (5ml, REMEL, UK) at 37^{o}C for 44 hours. Right after cytocholasin-B (Cyt-B, Sigma, Germany) was administrated and after 28 hours (total 72 hours of cultivation) the cells were collected by centrifugation (8 minutes, 800 rpm, room temperature). The cells were subjected to a cold hypotonic treatment, using 110 mM KCl (Fisher Bioreagents, Pittsburgh, PA, USA). After several washes with Carnoy solution (3:1 methanol to acetic acid) the samples were fixated with formaldehyde according to the protocol. Cell suspensions were dropped onto clean slides. Duplicate cultures and two slides per culture were prepared per donor. For observing the lymphatic cells and measuring the micronuclei two staining media were used. One slide of each culture was stained with Giemsa and the other slide was stained with DAPI. Slides with GIEMSA were stained for 20 min with freshly prepared 5% Giemsa in Sorensen buffer (pH 6.8; Prosan, Merelbeke, Belgium). After the staining, the staining media was removed with water and the slides were left to dry. DAPI staining had a final concentration of 0.17 μg/ml diluted in Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). In each slide two drops of 10 μl of the dye solution were placed and with a calyptrate (22mm x 50mm), it was spread to the surface of the slide. The slides with the two stainings were then stored in the dark at 4°C. Before reading them, they were left at room temperature for 30 minutes.

Slide scoring

Slide scoring and micronuclei enumeration in bi-nucleated T-lymphatic cells was performed by two different trained scientists according to the HUMN scoring criteria. (Human MicroNucleus Project) [242, 243]. The total number of bi-nucleated lymphatic cells was recorded in a specific data logger. Reading was performed by two ways:

1. By using a simple photonic microscope (DM 750, Leica Microsystems GmbH, Wetzlar, Germany),
2. By using the automated micronuclei counting system MetaSystems Metafer image cytometry system (metasystems, GmbH Altlussheim, Germany).

By using the simple photonic microscope for each slide the number of mono-nucleated, bi-nucleated and (MULTI) T-lymphatic cells (cells with 1, 2 or more cells respectively) in a total number of 2000 cells was recorded, using a mechanical counter (M.R.C. LTD, Holon, Israel). Cells with vague limits of cell plasma or deformed nuclei were not analyzed. Additionally in

each of the subcategories the number of cells with 1, 2 or more micronuclei in the cell plasma was recorded. For each sample thecytokinesis block proliferation index (CBPI) was calculated [CBPI = (1x MONO + 2x BN + 3x MULTI) / total number of cells and the frequency of micronuclei in 1000 bi-nucleated lymphatic cells].

Enumeration of Giemsa staining slides

The frequency of micronuclei using the slides with Giemsa staining with the photonic microscope in 40x magnification was calculated while the micronuclei distinction was performed by using 100x len (oil immersion lens). To avoid diversion of the standardized enumeration criteria 10 control samples that were occasionally enumerated to verify the enumeration competence of the user, were used.

Enumeration of DAPI staining slides

The enumeration of the slides with the DAPI staining was performed using the Metafer system. The distinction of the nuclei and micronuclei was performed using ultra violet radiation in 10 x magnifications and with special software (Metafer MN Score, Metasystems, Germany). With this method the presence of micronuclei in bi-nucleated lymphatic cells and the total of bi-nucleated lymphatic cells were estimated. The Metafer system can automatically recognize the bi-nucleated cells and the micronuclei in them allowing the user of the microscope to interfere with the scoring of the results as they appear. With this method we did not count mono-nucleated and (MULTI) T-lymphatic cells and we did not calculate CBPI. The Metafer system can read the whole sample by imprinting electronically the total bi-nucleated cells that it tracks and at the same time spot one or more micronuclei in them. After finishing the counting the researcher – user can interfere with the result. This stage is essential as it gives the opportunity to exclude the cells that do not conform to the scoring criteria and the estimation of the micronuclei in these cells. Additionally the cells are displayed in the computer screen allowing the researcher to observe in detail each optic area. An additional advantage of this system is the high analysis speed (approximately 8 slides / 90 minutes) and the user friendly Metafer software. Due to higher reliability and repeatability of the Metafer system we chose this system in order to analyze our results. We run our analysis using data from a minimum number of total bi-nucleated cells at 100 cells which resulted in 328 mother-child pairs.

Scoring criteria for micronuclei

Micronuclei were recognized by using the following scoring criteria of HUMN (Human MicroNucleus Project).

1. MN are morphologically identical to but smaller than the main nuclei.
2. The diameter of MN in human lymphocytes usually varies between 1/16 and 1/3 of the mean diameter of the main nuclei which corresponds to 1/256 and 1/9 of the area of one of the main nuclei in a BN cell, respectively.
3. MN are round or oval in shape.
4. MN are non-refractile and they can therefore be readily distinguished from artefacts such as staining particles.
5. MN are not linked or connected to the main nuclei.
6. MN may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.
7. MN usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

2.IV. Potential confounders

We examined the effect of potential confounding variables identified a priori in the literature. We considered the following variables as potential covariates. Maternal variables included maternal pre-pregnancy BMI (kg/m^2), maternal and paternal weight and height before pregnancy (kg, cm), weight gain during pregnancy (Kg), maternal age at birth (years), maternal and paternal ethnic origin (Greek, non-Greek), residence (urban, rural), marital status (married, not married), maternal working during pregnancy (yes, no), maternal and paternal work (unemployed, on payroll, self-employed, farming, unemployed looking for a job, unemployed due to medical reasons, student, on pension, other), parity (primiparous, multiparous), maternal and paternal educational level [low level: ≤ 9 years of mandatory schooling, medium level: > 9 years of schooling up to attending post- secondary school education and high level: attending university or having a university/technical college degree] and living in an urban area during pregnancy (yes, no). We tested if smoking during pregnancy is a potential confounder in four different manners. We tested smoking during early pregnancy (yes, no), smoking at 12 weeks

gestational age (yes, no), smoking at 30 week's gestational age (yes, no) and cotinine in mother's blood as a continuous variable. We also tested supplement intake during pregnancy (yes, no), folate intake during pregnancy (yes, no) and vitamin B12 intake during pregnancy (yes, no). Child variables included child's gender, exact age at outcome assessment, breastfeeding duration (months) and smoking during breastfeeding (yes, no), weight (Kgr), BMI (kg/m^2) and waist circumference (cm). Since there are no international WC percentile cut-off points defined to identify central adiposity in children or adolescents, we decided to use a Greek national reference to define central adiposity as waist circumference (WC) $\geq 90^{th}$ percentile for age and sex [244]. We categorized weight status at 4 years according to the International Obesity Task Force (IOTF) definitions [6] and we tested child's BMI categories as a potential confounder. We also tested attending a primary school before the age of 2 (yes, no), attending a primary school before the age of 4 (yes, no), exposure to passive smoking at the age of 4 (yes, no) and living in an urban area at the age of 4 (yes, no). Finally we tested if living near pollutionary areas (garage, farming areas, factory, power plant, antenna/radiator, sewer, airport, greenhouse, laboratory, stable, slaughter house, scrapheap, other pollutionary areas) or if residence area located near agrarian cultivations (yes, no) or proximity to agrarian cultivations (long distance >200m, close 50-200m, very close <50m) was a potential confounder.

2.V. Statistical analysis

Descriptive analyses of the study population characteristics, exposures and outcomes were conducted. Negative binomial regression models were used to estimate associations between $\log_{10}$ transformed POP exposures as continuous variables and as tertiles and micronuclei frequencies (MN per 1000 bi-nucleated cells) in childhood. We estimated incidence rate ratios (IRRs) and 95% confidence intervals (CIs).

Child's sex, maternal serum levels of triglycerides and total cholesterol were the covariates selected a priori for model adjustment. If inclusion of a variable (explained in detail above) altered the contaminant coefficient by $\geq 10\%$ or if they were independently associated with the outcome at $p < 0.10$, we retained the variable in the final set of covariates. The same set of covariates was used for all models. Final covariates included maternal pre-pregnancy BMI (kg/m^2), maternal age at birth (years), parity (primiparous, multiparous), maternal educational level [low level: ≤ 9 years of mandatory schooling, medium level: > 9 years of schooling up to

attending post- secondary school education and high level: attending university or having a university/technical college degree], smoking during early pregnancy, exposure to passive smoking and breastfeeding duration (months).

We looked for heterogeneity in associations related to pregnancy and to childhood variables by including interaction terms in the models and by stratifying the sample. Statistical significance was defined by an alpha level of 0.10 for interaction terms and of 0.05 for all other effect estimates. Thus we stratified the sample by maternal smoking at early pregnancy (yes, no), parity (primiparous, multiparous), maternal pre-pregnancy BMI status (<25 kg/m^2, ≥ 25 kg/m^2), child sex (male, female), breastfeeding (ever, never), breastfeeding duration (above/bellow 6 months), exposure to passive smoking (yes, no), residence area (urban, rural) and proximity to agrarian areas (yes, no).

We rerun our analysis using data from a minimum number of total bi-nucleated cells of 1000 cells which resulted in 145 mother-child pairs [57]. Analyses were conducted using STATA software, version 13.0 (Stata corp, College Station, TX).

3. RESULTS

3.I. Study population characteristics

Mothers, early childhood

A summary of the variables used in the analysis for the combined population (n = 328) of mothers and children with complete MN and POPs is presented in Table 1. Most of the participating mothers were Greek (93%), less than 35 years of age (82%) and before pregnancy 24% of mothers were overweight. Overall, women had a mean (± SD) age of 30.1 ± 4.9 years and a mean (± SD) of pre-pregnancy BMI of 24.9 ± 4.7 kg/m^2. Most mothers did not smoke and (64%) were married during pregnancy (86%), and had moderate education (52%). Almost all mothers (88%) initiated breastfeeding and the mean length of breastfeeding was 4.0 ± 4.2 months whereas nearly half of them self-reported smoking during lactation (42%).

Children (4 years of age)

The children had a mean (±SD) age of 4.2±0.3 years and half them were boys (52%). They had a mean (±SD) of height 105.1± 4.5 cm and a mean (±SD) of weight 18.1±2 Kg respectively. 79% of the children had a normal BMI while at the 4-year follow-up, the mean (± SD) offspring BMI was 16.3 ± 1.8 kg/m^2. In total, 45 (14%) children were classified as overweight and an additional 18 (5%) were obese at 4 years of age. Approximately half of them were exposed to passive smoking (44%). 70% lived in urban areas whereas 22% attended nursery school before the age of two and 81 % attended nursery school before the age of four (data shown in Table 2).

Missing data

All of the study covariates had missing data for some participants except child's gender. The percentage of missing values ranged from 0.3% (maternal age) to 12% (maternal serum levels of triglycerides and total cholesterol). We excluded from our final models mother-child pairs with missing data and we run our analysis with 264 mother-child pairs.

POP distribution among mothers

Blood concentrations of organochlorine pesticides and PCBs are presented in Table 3; an important finding is that all mothers included in this study had detectable levels of DDE, HCB, PCB 118 and PCB 180 indicating a generalized past exposure to these chemicals. Moreover, the

pesticide DDT, which is less persistent than its main metabolite (DDE), was detected in 34% of the study population. The highest concentrations were found for DDE, followed by total PCBs, HCB, and DDT. Spearman correlation coefficients (p-value) were 0.49 (<0.001) for DDE-PCBs, 0.47 (<0.001) for DDE-HCB, and 0.60 (<0.001) for PCBs-HCB.

MN frequency in children

Among children, the mean value of MN in peripheral blood lymphocytes was 2.50 ± 2.48 with the variation range from 0 to 16.08 MN/1000 BN while the median MN frequency was 1.87‰ in the 328 children (Table 4). Mann Whitney H test showed that there was a statistically significant difference in MN frequency between children of primiparous and multiparous mothers and between children exposed and non-exposed to passive smoking (Table 5). Children from primiparous mothers and children exposed to passive smoking had higher MN frequencies compared to children from multiparous women to children non-exposed to passive smoking .

3.II.Association of risk of MN formation and POPs

In children at the age of 4, increased risk of MN frequency per 1000 bi-nucleated cells was found for prenatal exposure to DDE and adjusted IRR per 10 fold increase was 1.47 (95% CI:1.05-2.06). Children of women in the highest tertile of exposure (1360.21-2734.08 pg/ml) had higher MNBN incidence (IRR $\log_{10}$ =1.4; 95%CI: 1.06, 1.96) compared to children of women in the low tertiles of exposure. Additionally when DDE concentration was categorized into tertiles, a linear trend was observed in the crude bivariate model, which tended to be stronger after adjusting for confounders (trend test p= 0.019).

No association was observed between in utero exposure to HCB and MN frequency per 1000 bi-nucleated cells (IRR 1.56 95% CI: 0.90, 2.72).

In crude analysis, higher levels of prenatal PCBs showed a non-significant positive association with micronuclei frequency per 1000 bi-nucleated T lymphatic cells at age 4. After adjusting for confounders, each 10-fold increase in total PCB levels was associated with higher MNBN incidence (IRR $\log_{10}$ =2.56; 95%CI: 1.34, 4.91). When PCBs concentration was

stratified by tertiles, we didn't observe a statistically significant risk for each of the exposure groups nor a dose response effect of prenatal exposure to PCBs to the micronuclei formation risk at the age of 4.

We found a positive association between MN formation and prenatal exposure to dioxin like PCBs (the sum of PCB 118 and 156) (IRR $\log_{10}$ =1.88; 95%CI: 1.03, 3.45) after adjusting for confounders. Micronuclei formation in peripheral T lymphatic cells at 4 years of age presented a positive trend in risk with increasing maternal non dioxin like PCBs levels (P trend = 0.008). For middle and high exposure groups to dioxin like PCBs there were increased risk for micronuclei formation in children IRR $\log_{10}$ = 1.54; 95%CI: 1.16, 2.05 and IRR $\log_{10}$ =1.60; 95%CI: 1.13, 2.26 respectively.

Prenatal exposure to non dioxin like PCBs (the sum of PCB 153, 138, 170 and 180) was also positively associated with MN formation (IRR $\log_{10}$ =2.57; 95%CI: 1.35, 4.89) in the fully adjusted models. When exposure to non-dioxin like PCBs concentration was stratified by tertiles, we didn't observe any linear trend nor a statistically significant risk of micronuclei formation for the in between exposure groups (Table 6).

3.III. Stratified analysis

We found no evidence of effect modification for micronuclei formation risk at 4 years (see Table 8 for DDE, Table 9 for HCB and table 10 for PCBs) by child sex, parity, smoking status during early pregnancy, pre-pregnancy BMI, exposure to passive smoking at the age of 4, and residence site (all p-interaction> 0.10). The association between prenatal HCB exposure and the MNBN incidence was stronger in children that breastfed (p-interaction 0.087).

Stratified analysis presented statistical significant risk for the micronuclei formation in the sub groups of primiparous women (DDE, HCB, PCBs), higher pre-pregnancy BMI (DDE), lower pre-pregnancy BMI (PCBs), girls (DDE, PCBs), non-exposure to passive smoking at the age of 4 (DDE,PCBs), ever initiated breast feeding (DDE, HCB, PCBs), breastfeeding duration <6 months (PCBs), living in an urban environment, near a garage or near an agrarian cultivation (PCBs) and living distant from an agrarian cultivation (DDE).

The review on the effect of smoking to the micronuclei frequency concluded that smoking should be measured as a continuous variable due to the possible U shape of effect on the micronuclei frequencies and the high impact of heavy smokers [245]. We run our analysis using maternal cotinine levels of mother at the 12^{th} week of gestation and our results presented the same pattern, though with higher IRRs and lower p values (data not shown, n=163).

3.IV. Sensitivity analysis

Sensitivity analyses excluding the micronuclei frequencies counted in children's peripheral T lymphatic cell in which we scored less than 1000 bi-nucleated cells (145 mother-child pairs in the adjusted model), did not meaningfully change our results for total PCBs, dioxin-like PCBs and non-dioxin like PCBs (see Table 7). The association of in utero exposure of fetuses to DDE and micronuclei formation at the age of 4 remained positive but not statistically significant for the continuous variable; the risk for each tertile of exposure was positive and also not statistically significant and additionally the dose-response effect was not apparent. Estimates were also not much different for HCB exposure, though a dose response manner for high versus middle exposure group was more obvious but still not statistically significant (trend test p= 0.061).

4. DISCUSSION

4.I. Brief summary of results

In the present study, we found that higher prenatal DDE and PCBs levels were associated with increased MN frequencies at preschool age. We found no association between HCB levels in pregnancy and child MN frequencies. This is the first study to our knowledge that prospectively evaluated the impact of pregnancy exposure to organochlorine compounds and MN formation in children and confirmed the higher risk in MN formation due to prenatal POP exposure.

4.II. Comparison to other studies

The MN assay has been used to study genome damage in children after trans placental and postnatal exposures in a variety of rural or urban environmental settings as well as from accidental overexposures [62]. Up to date potentially genotoxic factors include tobacco smoke, airborne nanoparticles, food contaminants (POPs and chemicals generated by overcooking) non-ionizing and ionizing radiation. The Rhea birth cohort studied such exposures and found increased MN frequencies in pregnant women exposed to airborne contaminants [246] and water contaminants (brominated trihalomethanes) [247].

The hypothesis that DDT/DDE, HCB and PCBs induce MN formation has been explored in previous studies. A study in Mexico suggested that prenatal exposure to DDT causes adverse effects in newborns and mothers but findings did not confirm this assumption [236]. Prenatal exposure to PCBs was not associated with increased MN frequencies in newborns [237] and exposure of school children and their mothers to PCBs also was not associated with MN formation [63]. Our study is the first to confirm prenatal exposure to DDE and PCBs and increased risk for micronuclei formation in children at the age of 4.

The MN test allows the identification of alterations that are formed after the cell has undergone DNA repair, synthesis, and mitosis in culture. These alterations are related to chromosome breakage, the formation of translocations or to malsegregation during anaphase, which are finally observed as micronuclei, chromatin buds, or nucleoplasmic bridges [225, 248, 249]. Thus the MN assay measures damage only in surviving cells and cells eliminated by apoptosis do not contribute to the estimated frequencies. Given that circulating T lymphocytes

are thought to accumulate MN over several months or even years, after which cells with abnormalities disappear except if permanent mutations are present in stem cells [62], the results of the present study suggest that the early prenatal period is a critical period for genotoxic events caused by DDE and PCBs.

Cross sectional studies in human populations found that umbilical cord (UC) blood cells carry a greater burden of POPs in comparison to maternal blood [234, 236] and almost systematically score lesser micronuclei frequencies from the mothers [70, 237]. Interestingly among newborns, risks from prenatal POP exposures are not apparent [236]. Maybe this is because UC cells have undergone greater cell death [236, 250] and the MN assay is not suitable to detect the effect (too many apoptotic cells). A strength of our prospective analysis in addition to our measured POP concentrations is that we were able to detect increased risks spanning the prenatal exposure and perhaps the combined prenatal and postnatal period.

After birth the UC will end its purpose while the child will carry the ontogenesis. Data from a birth cohort in Spain, shows that the burden DDE/HCB and PCBs from placenta to adolescence is ongoing and picks in early childhood [251]. This means that the child will carry also the possible outcomes of this ongoing exposure and with our study we suggest that they become observed at age of 4.

Taken as a whole our findings suggests that the association between PCB and DDE and possible genotoxic events are time- and dose-specific, rather than only dose-specific. We can assume that our positive association findings would be more obvious if we had measured prenatal exposures to POPs and MN frequencies at much older ages prospectively. Epidemiologic data are also consistent with this hypothesis. Results from a large pregnancy cohort associate prenatal DDT and postpartum PCB 203 with the risk of breast cancer diagnosis at the age of 50 [104, 139]. Additionally case control studies associate PCB and HCB exposure in utero and men with testicular cancer [131, 232].

The switching of these associations (evident in prospective studies and not evident in cross sectional research) is controlled to a great extent by age. In children at 1–4 years of age the MN are increased by 66% due to age [252]. It is possible that our presented risk with POPs during gestation is observed because there is a critical exposure window at the first trimester of pregnancy or it is observed because a cumulative effect was detectable after 4 years of age, or

both. Also developmental changes in response to POPs that are hazardous to the genome, are currently not well understood [62].

In vitro exposures of DDT on human cells and scoring the frequency of micronuclei, introduces DDT to be weakly genotoxic [74, 75, 253]. Further cytogenetic studies in rats suggest that DDT shows a hormetic effect in ROS formation after administration of more than 20 ppb. Under this low-dose threshold most carcinogenic indications were not observed implying an effective DNA- repair capacity. Above this threshold a dose-dependent induction of anti-oxidant activity was observed in hepatic cells [254]. In concordance exposure to low doses of DDT induced hepatocellular carcinoma through oxidative stress [122]. Although the epidemiological data were not consistent for liver cancer and DDT as some reported dose-response [107-109], one reported positive [38] and two others reported no association [38, 110], our findings are similar to the cytogenetic findings of DDT in liver cells as a more marked association is observed in high exposure tertile and MN formation.

The possible genotoxic potential of HCB in vitro by using MN frequencies has not been confirmed [253] and this consistent with the finding of our study that did not observe any association between prenatal exposure to HCB and MN frequencies in preschool children.

Previous in vitro experiments using human cells exposed to PCB126 [152, 255, 256] and PCB 153 [152, 255, 256] have shown that they could effectively increase MN formation. Consistent associations were observed between PCB-induced changes in the levels of cellular reactive oxygen species (ROS) and DNA damage resulting in cytotoxicity (MNBN). A dose-dependent increase in micronuclei frequency was observed in PCB-153 cells, consistent with an increase in histone 2AX phosphorylation [257]. These findings are in concordance with our positive associations of PCBs exposure and MN formation in children.

A meta-analysis of epidemiologic data has shown that PCBs contribute to non-Hodgkin's lymphoma [84, 133]. Additionally children's exposure to PCBs through PCB 118, 138, and 153 in household dust has been associated with 2 fold risk of leukemia in the findings of a meta-analysis by Ward et al in 2009 [113]. There's equivocal conclusions on prostate cancer [134-137], breast cancer [106, 133, 138] and PCBs and associations between DDT, non-Hodgkin's lymphoma [76-95], leukemia [81, 87, 90] prostate cancer [97-101], breast cancer [102-106] liver cancer [38, 110, 111] and testicular cancer [38, 112]. For many cancer types and POP exposures there are uncovered areas where no research has yet been conducted, which could direct future

studies with the systematic review process. It is important to note that it is the genetic damage induced in vivo, which is not repaired, that is of interest when measuring the micronuclei in the binucleated lymphocytes. The genetic damage that is transmissible through cell division and therefore will be detected in the MN assay is the damage that may lead to the increased risk and association with later cancer incidences [43, 48]. Cancers incidence was found significantly higher in groups with medium and high MN frequency [48] and our results indicate a high risk of MN formation due to prenatal DDE and PCBs.

4.III. RISK FACTORS FOR MICRONUCLEI FORMATION

Our estimated MN frequencies were low as expected for children [64]. Age alone contributes to micronuclei frequencies according to findings from previous studies in adults [65, 258-261], without presenting seasonal variances in a year's time [262]. We rerun our final models including child's exact age as an effect modifier and this was verified (data not shown). There was no evidence in our study that MN frequency differs among boys and girls similarly to previous findings [70, 263, 264].

Our results showed that children that have been breastfed presented higher risk for MN formation associated to all POPs exposure compared to children that never breastfed. This finding suggests that the major exposure to POPs occurs through diet and food with high lipid content as previously reported [251]. Additionally in 3 year old children, prolonged breastfeeding duration was a major determinant of increased PCBs concentrations [265].

Cohort studies found that low parity, is one of the most important determinant of increased plasma concentrations of PCBs in pregnant women [265, 266]. As the parity increases, the contaminant concentrations of DDE tended to decrease [266]. At the same time in our stratified analysis children of primiparous women presented higher risk of MN formation in association to prenatal DDE, PCBs and HCB.

Also low pre-pregnant BMI increases plasma concentrations of PCBs in pregnant women [265, 266]. We found that children from mothers with low BMI presented risk in MN formation due to prenatal PCBs exposure and this also confirms our dose response findings as women with lower pre-pregnancy BMI carry higher PCB_S burden according to the previous studies.

A meta-analysis of chromosomal damage evaluated with the CBMN assay subsequent to pesticide exposure, presented that exposed subjects had significantly higher MN frequencies than

controls [267]. Another study that used geographic information systems (GISs) to explore possible associations between MN in children and their mothers and traffic [268], suggested a greater vulnerability to traffic-related air pollution in children and finally children from pollution events with PCBs also presented higher MN frequencies [269]. We explored the MN formation risk in children living near different pollutionary areas, to see if an effect was more observed. DDT prenatal exposure associated in high risk formation in children living away from agrarian cultivations, HCB associated with MN formation in children of an urban environment and PCBs associated with MN formation in children living near a garage or an agrarian cultivation (data presented in tables 8, 9, 10 respectively).

4.IV. Biological mechanism

Recently there was no broadly accepted method to systematically organize and evaluate mechanistic data on human carcinogens; data that are key to assessing the potential carcinogenicity of unstudied chemicals [270]. Researchers applied a new method to create order among a sprawling body of mechanistic data, based on 10 key characteristics of human carcinogens as follows: 1) act as an electrophile either directly or after metabolic activation; 2) be genotoxic; 3) alter DNA repair or cause genomic instability; 4) induce epigenetic alterations; 5) induce oxidative stress; 6) induce chronic inflammation; 7) be immunosuppressive; 8) modulate receptor-mediated effects; 9) cause immortalization; and 10) alter cell proliferation, cell death, or nutrient supply [271].

DDT was associated with 3 characteristics [38]. Evidence of DDT affecting immunosuppression is numerous [114-117] and evidence of increasing oxidative stress in human peripheral blood mononuclear cells [118, 119, 121, 122]. The term “epigenetic” refers to stable changes in gene expression and chromatin organization that are not caused by changes in the DNA sequence itself and can be inherited over cell divisions. Epigenetic phenomena, including changes to the DNA methylome and chromatin compaction states, along with histone modification can impact the carcinogenic process by affecting gene expression and DNA repair dynamics [272]. Ruiz-Hernandez et al recently investigated the bibliography of endocrine disruptors on DNA methylation [9] and noticed a trend towards hypomethylation with increasing levels of exposure to DDT [126, 128, 129]. DNA methylation was lower in 9-year olds compared

to newborns and was higher in boys compared to girls. These data suggest that repeat element methylation can be an informative marker of epigenetic differences by age and sex and that prenatal exposure to POPs may be linked to hypomethylation in fetal blood [130].

PCBs can induce formation of reactive oxygen species, are genotoxic, cause immune suppression, induce an inflammatory response, and modulate receptor-mediated effects. Dioxin-like congeners act primarily through activation of the aryl hydrocarbon (Ah) receptor and subsequent events, while lower-chlorinated congeners begin through metabolic activation [144] as reviewed recently [273]. These constitute a majority of the key characteristics as described earlier.

Direct mechanisms by which chemical genotoxins can cause structural chromosome aberrations or numerical chromosome changes mainly include the formation of small or bulky adducts, DNA strand cross-links, DNA-protein cross-links and DNA strand breaks. Indirect mechanisms include inhibition of DNA repair, impairment of chromosome segregation, disruption of mitotic checkpoints machinery, inhibition of apoptosis, perturbation of cytokinesis, inhibition of enzymes involved in the maintenance of DNA methylation, and induction of inflammation and/or mitochondrial dysfunction leading to increased oxidative [43]. All these mechanisms may result in MN formation. DDE and PCBs maybe possible genotoxins through indirect mechanisms such as DNA hypomethylation and ROS formation at early exposures.

4.V. Strength and limits

Our analysis has its limitations. The inclusion of MN frequency in prospective cohort studies has been very limited and a generalized conclusion with the use of MN as end point should be addressed cautiously. Whether the MN formation is true because prenatal POP exposures or the intra-individual variability of the measure or the association with the most recent exposure, or disease under consideration is difficult to determine [60]. Nevertheless we observed strong positive associations between prenatal exposures to DDT and dioxin like PCBs and high risk of MN formation in children appeared with the same pattern after our sensitivity and stratification analysis and also cytogenetic studies that present the same patterns. A second limitation is that we did not control for gestational age and previous exposure to POPs through diet with exception breastfeeding.

Our strong advantage is the prospective design of the study and the sufficient number of mother-child pairs with the primary focus to explore POP's fetal exposure in early pregnancy and micronuclei formation in the age of 4. MN data for children that should help establish the predictive value of MN not only for cancer but possibly for other health conditions of childhood and adulthood. It would require large prospective studies combining exposure data (beginning from pregnancy and during childhood) with comprehensive assessment of lifestyle factors and health status [62]. This was proposed by Holland et al 2011 in the review of Micronuclei frequencies in children. The reasons were mainly because T lymphocytes to be analysed by the MN assay are circulating in peripheral blood for only 6 months, genome stability during foetal life might be different than later in ontogenesis, baseline MN frequency is relatively low and cell proliferation may vary in children by age and in different cell types. Micronuclei induction in newborns were not associated with prenatal POPs and in our analysis micronuclei formation in 4 years of age, presented risk. This information is important to the timing of the cell collection to assess exposure of genotoxicity events. Additionally and we examined thoroughly the impact of demographic parameters such as age, gender, smoking and other lifestyle factors in our evaluation.

4.VI. Conclusion

All mothers had detectable levels of organochlorine pesticides and PCBs in their blood indicating a generalized past exposure. With our study we provide new evidence that exposure to DDE and PCBs during pregnancy may increase the risk of micronuclei formation in children. To our knowledge this is the first study to associated prenatal POPs exposure and MN formation in children and further studies are needed to confirm these findings and examine the biological mechanisms underlying the observed associations. Further follow up of this cohort will allow determining if prenatal exposure to POPs has, in addition, an effect on genetoxicity in late childhood and also long term carcinogenic risks.

Additional studies are required to evaluate possible associations between MN frequencies in age periods of children and the re-evaluate the impact of prenatal exposures to POPs in each time series in a cohort, in order to elucidate time windows of genotoxic effects. Possible biological mechanisms of MN formation in children due to POP exposures attend further elucidation. MN frequencies in children may or may not cause a direct correlation of cancer incidence and may or

may not postulate a high risk of cancer development in their adulthood and this questions need also to be addressed.

5. TABLES

Table 1. Characteristics of study population at pregnancy and infancy (n=328).

Characteristic	N	Value[a]
Pregnancy		
Age (years)	327	30.1 ± 4.9
Pre-pregnancy BMI (kg/m2)	320	24.9 ± 4.7
Overweight/ Obesity		
Underweight (<18.5)	6	1.8
Normal (≥18.5-25)	192	58.5
Overweight (≥25-30)	80	24.4
Obese (≥30)	42	12.8
Marital status		
Married	282	86
Other	41	12.5
Ethnic origin		
Other	23	7
Greek	305	93
Education		
Low	48	14.6
Medium	171	52.1
High	106	32.3
Parity		
Primiparous	145	44.2
Multiparous	182	55.5
Smoking during pregnancy		
Never	209	65.3
Ever	111	34.9
Working during pregnancy		
No	172	52.4
Yes	148	45.1
Breastfeeding (months)	316	4.0 ± 4.2
Breastfeeding		
Never	38	12
Ever	278	88
Breastfeeding duration		
Breastfeeding< 6 months	224	71
Breastfeeding ≥ 6 months	92	29
Smoking during breastfeeding		
Never	174	58
Ever	126	42

[a] Values are Mean ± SD or % if not indicated otherwise.

Table 2. Characteristics of study population at early childhood (4 year old) (n=328).

Characteristic	N	Value [a]
Childhood (4 years)		
Age	328	4.2 ± 0.3
Sex		
Boy	169	51.5
Girl	159	48.5
Weight	328	18.1 ± 2.0
Height	328	105.1 ± 4.5
BMI	328	16.3 ± 1.8
Overweight/ Obesity		
Underweight	7	2.1
Normal	258	78.7
Overweight	45	13.7
Obese	18	5.5
Exposure to passive smoking		
No	175	53.4
Yes	143	43.6
Residence Site		
Urban	231	70.4
Rural	97	29.6
Attendance to nursery school		
< 2 years	73	22.3
< 4 years	265	81.3

[a] Values are Mean ± SD or % if not indicated otherwise.

Table 3. Concentrations of POPs measured in 1st trimester maternal serum (pg/ml, n = 328)

				Percentile			
Contaminants	**GM (95% CI)**	**min**	**max**	**25th**	**50th**	**75th**	**% above LOQ[a]**
DDT	40.4 (37.4, 43.8)	25	1093	25	25	62.8	34.1
HCB	87.8 (82.7, 93.6)	19.5	1330.5	59.9	81.1	118.7	100
DDE	1960.1 (1789, 2150)	189.8	23175.3	1081.5	1992.1	3479.1	100
PCB118	17.2 (16.1, 18.4)	3b	144	12.1	18.1	25.3	96.6
PCB153	124.6 (116.3, 132.5)	24.9	707.1	86.3	124.6	188.8	100
PCB138	65.9 (61.4, 70.1)	5 b	281.6	44.8	65.4	99.5	99.7
PCB156	6.1 (5.6, 6.6)	3 b	47	3 b	6.7	11	55.5
PCB180	67.9 (61.4,70.1)	13.1	670.3	44.3	66.5	106.6	100
PCB170	34.1 (31.5, 36.7)	5 [b]	275.5	23.1	34.1	54.9	97
BDE47	6.8 (6.3, 7.3)	5 [b]	168	5 [b]	5 [b]	5 [b]	22.3
Sum of non dioxin like PCBs	225.3 (275.5, 314.5)	59.8	1927.9	201.5	290.8	449.6	
Sum of dioxin like PCBs	24.2 (22.7, 25.8))	6 b	191	16.6	24.2	63.4	
Total PCBs	320.9 (299.6, 341.5)	66.2	1988.4	218.5	319.4	478.4	

HCB, hexachlorobenzene; DDE, dichlorodiphenyl dichloroethene; DDT, dichlorodiphenyl trichloroethane; PCB polychlorinated biphenyl, six individual PCB congeners (IUPAC numbers: 118, 138, 153, 156, 170 and 180); BDE-47, tetra-bromodiphenyl ether; Sum of non dioxin like PCBs, sum of PCB 153, 138, 170 and 180; Sum of dioxin like PCBs, sum of PCB 118 and 156; total PCBs, sum of PCB concentrations.

[a] LOQ was 6 pg/ml for PCB118 and PCB 156; 10 pg/ml for HCB, DDE, PCB138, PCB153, PCB170, and PCB180. [b] Value is LOQ/2.

Table 4. Mean, median, interquartile range, min, max of micronuclei frequency in 4 year's old children (n = 328)

	N	Mean (SD)	Median	IQR	Min	Max
No BN	328	1496.0 (1345.7)	1102.0	1530	101	6492
MN1000BN (‰)	328	2.50 (2.48)	1.87	2.23	0	16.08

MN, micronuclei; BN, bi-nucleated T lymphocytes; MN1000BN, MN per 1000 bi-nucleated T lymphocytes; IQR, interquartile range

Table 5. Micronuclei frequency in 4 year's old children by study population characteristics (n = 328)

Characteristic	N	MN1000BN (‰) Value[a]
Pregnancy		
Maternal age at birth		
≥16-27	74	2.44 (2.42)
≥27-30	61	2.11 (2.34)
≥30	192	2.62 (2.54)
Overweight/ Obesity		
Pre-pregnancy BMI (kg/m2)		
Underweight (<18.5)	6	1.96 (1.45)
Normal (≥18.5-25)	192	2.76 (2.79)
Overweight (≥25-30)	80	2.15 (1.85)
Obese (≥30)	42	1.80 (1.57)
Marital status		
Married	282	2.46 (2.54)
Other	41	2.50 (1.90)
Ethnic origin		
Other	23	2.71 (1.94)
Greek	305	2.48 (2.52)
Education		
Low	48	2.39 (1.90)
Medium	171	2.33 (2.29)
High	106	2.74 (2.90)
Parity [b]		
Primiparous	145	2.94 (2.74)
Multiparous	182	2.13 (2.21)
Smoking during early pregnancy		
Never	209	2.39 (2.37)
Ever	111	2.64 (2.64)
Working during pregnancy		
No	172	2.66 (2.62)
Yes	148	2.27 (2.27)
Child characteristics		
Sex		
Boy	169	2.57 (2.54)
Girl	159	2.42 (2.43)
Age		

≤ 4,5 years	282	2.60 (2.60)
> 4,5 years	46	1.86 (1.46)
Breastfeeding		
Never	38	1.81 (1.54)
Ever	278	2.59 (2.61)
Breastfeeding duration		
Breastfeeding< 6 months	224	2.52 (2.51)
Breastfeeding ≥ 6 months	92	2.45 (2.53)
Smoking during breastfeeding		
Never	174	2.36 (2.43)
Ever	126	2.63 (2.55)
Overweight/ Obesity		
Underweight	7	4.06 (3.69)
Normal	258	2.38 (3.39)
Overweight	45	2.77 (2.95)
Obese	18	2.86 (1.76)
Exposure to passive smoking [b]		
No	175	2.75 (2.66)
Yes	143	2.18 (2.27)
Residence Site		
Urban	231	2.44 (2.26)
Rural	97	2.52 (2.57)
Residence Site [b]		
Distant from agrarian cultivation	214	2.60 (2.60)
Near agrarian cultivation	92	2.23 (2.32)

MN, micronuclei; BN, bi-nucleated T lymphocytes; MN1000BN, MN per 1000 bi-nucleated T lymphocytes

[a] Values are Mean ± SD or % if not indicated otherwise.

[b] A Mann Whitney H test showed that there was a statistically significant difference in MN1000BN between the groups $p = 0.05$

Table 6. Associations of first trimester maternal serum POP levels with micronuclei frequency at 4 years of age, mother-child cohort, Crete, Greece.

	MN1000BN (n=328)		MN1000BN (n=264)	
	Crude		Adjusted[a]	
	IRR (95% CI)	p-value	IRR (95% CI)	p-value
DDE log_{10} pg/ml	1.31 (1.00, 1.71)	**0.050**	1.47 (1.05, 2.06)	**0.024**
Low	1		1	
Middle	1.18 (0.92, 1.50)	0.199	1.24 (0.93, 1.66)	0.170
High	1.29 (1.01, 1.65)	**0.041**	1.44 (1.06, 1.96)	**0.023**
Trend test	**0.042**		**0.019**	
HCB log_{10} pg/ml	1.16 (0.77, 1.77)	0.487	1.56 (0.90, 2.72)	0.113
Low	1		1	
Middle	1.01 (0.79, 1.29)	0.947	1.07 (0.80, 1.43)	0.647
High	1.17 (0.92, 1.49)	0.209	1.29 (0.94, 1.76)	0.149
Trend test	0.205		0.145	
PCBs log_{10} pg/ml	1.39 (0.94, 2.05)	0.095	2.56 (1.34, 4.91)	**0.005**
Low	1		1	
Middle	1.08 (0.85, 1.38)	0.536	1.11 (0.82, 1.49)	0.510
High	1.13 (0.88, 1.44)	0.327	1.15 (0.81, 1.65)	0.427
Trend test	0.328		0.430	
Dioxin like PCBs log_{10} pg/ml	1.24 (0.83, 1.86)	0.300	1.88 (1.03, 3.45)	**0.040**
Low	1		1	
Middle	1.34 (1.05, 1.71)	**0.020**	1.54 (1.16, 2.05)	**0.003**
High	1.27 (1.00, 1.63)	0.055	1.60 (1.13, 2.26)	**0.008**
Trend test	**0.063**		**0.008**	
non Dioxin like PCBs log_{10} pg/ml	1.39 (0.95, 2.03)	0.092	2.57 (1.35, 4.89)	**0.004**
Low	1		1	
Middle	1.08 (0.84, 1.38)	0.545	1.05 (0.77, 1.42)	0.764
High	1.13 (1.13, 0.88)	0.338	1.09 (0.77, 1.56)	0.620
Trend test	0.339		0.620	

MN, micronuclei, BN, bi-nucleated T lymphocytes; MN1000BN, MN per 1000 bi-nucleated T lymphocytes; DDE tertiles: low (189.85-1342.44 pg/ml), middle (1360.21-2734.08 pg/ml), and high (2738.37-23175.4 pg/ml), HCB tertiles: low (19.52-68.63 pg/ml), middle (68.71-101.19 pg/ml), and high (102.49-1330.51 pg/ml), PCBs tertiles: low (66.23-247.45 pg/ml), middle (247.62-412.19 pg/ml), and high (413.37-1988.38 pg/ml), Dioxin like PCBs tertiles: low (6-18.95 pg/ml), middle (19.13-30.51 pg/ml), and high (30.62-190.68 pg/ml), non Dioxin like PCBs tertiles: low (59.78-226.94 pg/ml), middle (227.28-383.05 pg/ml), and high (386.83-1927.95 pg/ml).

[a]Adjusted for child's sex (boy, girl), child's exposure to passive smoking (yes, no), maternal age (continuous variables), smoking during early pregnancy (yes, no), breastfeeding duration (months), pre-pregnancy BMI (kilograms per meter squared), parity (primiparous, multiparous), maternal educational level [low (≤ 9 years of mandatory schooling), medium (> 9 years of schooling up to attending postsecondary school education), high (attending university or having a university/technical college degree)] and maternal serum triglycerides and cholesterol.

Table 7. Associations of first trimester maternal serum POP levels with micronuclei frequency at 4 years of age if bi-nucleated T lymphocytes >1000, mother-child cohort, Crete, Greece.

	MN1000BN (n=175)		MN1000BN (n=145)	
	Crude		Adjusted [a]	
	IRR (95% CI)	p-value	IRR (95% CI)	p-value
DDE log_{10} pg/ml	1.21 (0.90, 1.64)	0.204	1.22 (0.84, 1.76)	0.302
Low	1		1	
Middle	1.22 (0.94, 1.59)	0.143	1.25 (0.91, 1.71)	0.169
High	1.16 (0.88, 1.53)	0.280	1.10 (0.78, 1.57)	0.581
Trend test	0.279		0.583	
HCB log_{10} pg/ml	1.14 (0.77, 1.70)	0.516	1.64 (0.95, 2.83)	0.075
Low	1		1	
Middle	0.99 (0.76, 1.30)	0.953	1.09 (0.79, 1.50)	0.607
High	1.14 (0.87, 1.49)	0.322	1.38 (0.97, 1.97)	0.077
Trend test	0.326		0.068	
PCBs log_{10} pg/ml	1.33 (0.88, 2.03)	0.179	2.58 (1.27, 5.21)	**0.009**
Low	1		1	
Middle	1.15 (0.87, 1.50)	0.321	1.17 (0.83, 1.64)	0.366
High	1.20 (0.91, 1.58)	0.198	1.38 (0.92, 2.06)	0.121
Trend test	0.203		0.119	
Dioxin like PCBs log_{10} pg/ml	1.36 (0.87, 2.12)	0.176	2.06 (1.05, 4.03)	**0.034**
Low	1		1	
Middle	1.39 (0.94, 1.63)	0.122	1.26 (0.90, 1.75)	0.173
High	1.24 (0.94, 1.63)	0.128	1.45 (0.98, 2.14)	0.060
Trend test	0.138		0.061	
non Dioxin like PCBs log_{10} pg/ml	1.32 (0.87, 1.99)	0.191	2.53 (1.26, 5.08)	**0.009**
Low	1		1	
Middle	1.06 (0.81, 1.39)	0.668	1.05 (0.74, 1.47)	0.790
High	1.14 (0.87, 1.50)	0.339	1.25 (0.83, 1.87)	0.284
Trend test	0.338		0.266	

MN, micronuclei; BN, bi-nucleated T lymphocytes; MN1000BN, MN per 1000 bi-nucleated T lymphocytes; DDE tertiles: low (189.85-1342.44 pg/ml), middle (1371.29-2685.69 pg/ml), and high (2738.37-19975.4 pg/ml),HCB tertiles: low (46.43-68.63 pg/ml), middle (69.71-101.19 pg/ml), and high (102.77-1060.75 pg/ml), PCBs tertiles: low (66.23-247.43 pg/ml), middle (248.31-412.19 pg/ml), and high (413.37-1788.82 pg/ml), Dioxin like PCBs tertiles: low (6-18.86 pg/ml), middle (19.13-30.39 pg/ml), and high (30.62-190.68 pg/ml), non-Dioxin like PCBs tertiles: low (59.78-226.94 pg/ml), middle (227.28-383.05 pg/ml), and high (386.83-1690.75 pg/ml).

[a] Adjusted for child's sex (boy, girl), child's exposure to passive smoking (yes, no), maternal age (continuous variables), during early pregnancy (yes, no), breastfeeding duration (months), pre-pregnancy BMI (kilograms per meter squared), parity (primiparous, multiparous), maternal educational level [low (≤ 9 years of mandatory schooling), medium (> 9 years of schooling up to attending postsecondary school education), high (attending university or having a university/technical college degree)] and maternal serum triglycerides and cholesterol.

Table 8. Associations of first trimester maternal serum DDE (log_{10} pg/ml) levels with micronuclei frequency at 4 years of age in the subgroups defined by child's gender, smoking status during pregnancy, parity, breastfeeding, maternal pre pregnancy BMI status, passive smoking at the age of 4, residence area, mother-child cohort, Crete, Greece.

		MN1000BN
Characteristic	**N**	**IRR (95% CI)**
Child's Sex		
Boy	131	1.32 (0.81, 2.14)
Girl	133	1.60 (1.00, 2.57)
p-interaction		0.644
Smoking during pregnancy		
Non-smokers at early pregnancy	179	1.24 (0.82, 1.87)
Smokers at early pregnancy	85	1.72 (0.84, 2.77)
p-interaction		0.204
Breastfeeding		
Never breast feeding	30	0.68 (0.21, 2.17)
Ever breastfeeding	234	1.58 (1.1, 2.45)
p-interaction		0.117
Breastfeeding duration		
Breastfeeding <6 months	186	1.51 (0.98, 2.32)
Breastfeeding ≥ 6 months	78	1.21 (0.70, 2.09)
p-interaction		0.851
Parity		
Primiparous	109	2.13 (1.30, 3.77)
Multiparous	155	1.28 (0.82, 1.87)
p-interaction		0.231
Pre-pregnancy BMI (kg/m2)		
	1	
	5	
Pre-pregnancy BMI <25 kg/m^2	7	1.23 (0.77, 1.96)
Pre-pregnancy BMI ≥25 kg/m^2	107	2.18 (1.36, 3.50)
p-interaction		0.283
Exposure to passive smoking		
No	143	1.30 (0.83, 2.03)
Yes	121	1.32 (0.79, 2.20)
p-interaction		0.660
Residence site		
Urban	188	1.44 (0.97, 2.13)
Rural	76	1.44 (0.74, 2.82)
p-interaction		0.941
Residence site		
Distant from agrarian cultivation	174	1.53 (1.02, 2.30)
Near agrarian cultivation	80	1.00 (0.50, 2.31)

p-interaction	0.258

MN, micronuclei; BN, bi-nucleated T lymphocytes; MN1000BN, MN per 1000 bi-nucleated T lymphocytes; DDE, dichlorodiphenyl dichloroethene

Table 9. Associations of first trimester maternal serum HCB (log_{10} pg/ml) levels with micronuclei frequency at 4 years of age in the subgroups defined by child's gender, smoking status during pregnancy, parity, breastfeeding, maternal pre pregnancy BMI status, passive smoking at the age of 4, residence area, mother-child cohort, Crete, Greece.

		MN1000BN
Characteristic	**N**	**IRR (95% CI)**
Child's Sex		
Boy	133	1.05 (0.42, 2.61)
Girl	131	1.96 (0.97, 3.98)
p-interaction		0.720
Smoking during pregnancy		
Non-smokers at early pregnancy	179	1.70 (0.83, 3.44)
Smokers at early pregnancy	85	1.15 (0.47, 2.84)
p-interaction		0.668
Breastfeeding		
Never breast feeding	30	0.69 (0.20, 2.38)
Ever breastfeeding	234	2.06 (1.08, 3.91)
p-interaction		0.087
Breastfeeding duration		
Breastfeeding <6 months	186	1.56 (0.78, 3.14)
Breastfeeding ≥ 6 months	78	1.32 (0.51, 3.43)
p-interaction		0.989
Parity		
Primiparous	109	3.l2 (1.21, 8.08)
Multiparous	121	1.25 (0.71, 2.62)
p-interaction		0.557
Pre-pregnancy BMI (kg/m2)		
Pre-pregnancy BMI <25 kg/m^2	157	1.56 (0.77, 3.13)
Pre-pregnancy BMI ≥25 kg/m^2	107	1.46 (0.55, 3.86)
p-interaction		0.820
Exposure to passive smoking		
No	143	2.17 (1.11, 4.25)
Yes	127	0.71 (0.29, 1.75)
p-interaction		0.304
Residence site		
Urban	188	1.15 (0.61, 2.15)
Rural	76	3.42 (1.14, 10.32)
p-interaction		0.180
Residence site		
Distant from agrarian cultivation	174	1.82 (0.89, 3.73)
Near agrarian cultivation	80	0.91 (0.33, 2.54)
p-interaction		0.436

MN, micronuclei; BN, bi-nucleated T lymphocytes; MN1000BN, MN per 1000 bi-nucleated T lymphocytes; HCB, hexachlorobenzene

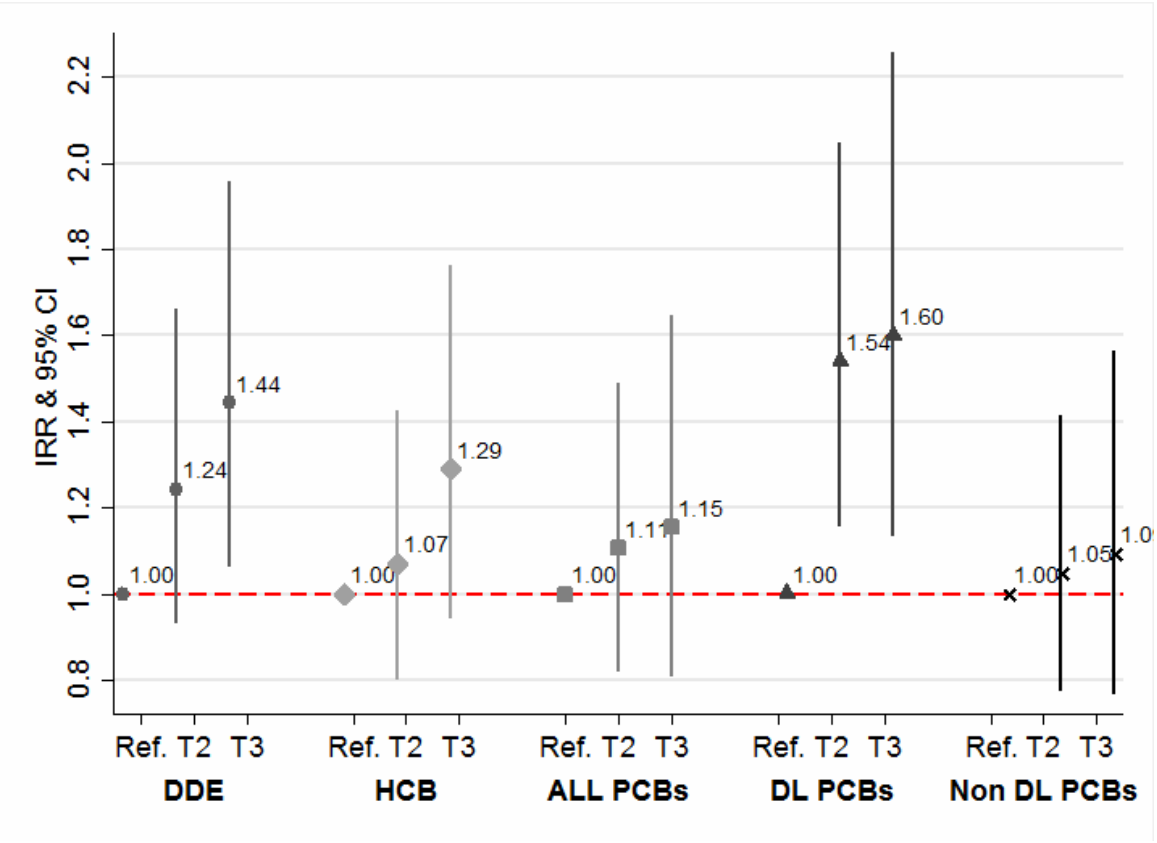
Table 10. Associations of first trimester maternal serum PCBs (log_{10} pg/ml) levels with micronuclei frequency at 4 years of age in the subgroups defined by child's gender, smoking status during pregnancy, parity, breastfeeding, maternal pre pregnancy BMI status, passive smoking at the age of 4, residence area, mother-child cohort, Crete, Greece.

		MN1000BN
Characteristic	**N**	**IRR (95% CI)**
Child's Sex		
Boy	133	2.67 (1.07, 6.69)
Girl	131	2.59 (1.04, 6.46)
p-interaction		0.720
Smoking during pregnancy		
Non-smokers at early pregnancy	179	2.01 (0.94, 4.30)
Smokers at early pregnancy	85	2.38 (0.61, 9.26)
p-interaction		0.247
Breastfeeding		
Never breast feeding	30	1.79 (0.19, 16.65)
Ever breastfeeding	234	2.67 (1.33, 5.33)
p-interaction		0.230
Breastfeeding duration		
Breastfeeding <6 months	186	4.38 (1.91, 10.04)
Breastfeeding ≥ 6 months	78	0.95 (0.32, 2.80)
p-interaction		0.230
Parity		
Primiparous	109	3.56 (1.17, 10.88)
Multiparous	155	1.97 (0.86, 4.50)
p-interaction		0.543
Pre-pregnancy BMI (kg/m2)		
Pre-pregnancy BMI <25 kg/m^2	157	2.79 (1.14, 6.85)
Pre-pregnancy BMI ≥25 kg/m^2	107	1.86 (0.73, 4.74)
p-interaction		0.570
Exposure to passive smoking		
No	143	2.84 (1.26, 6.43)
Yes	121	0.96 (0.30, 3.07)
p-interaction		0.520
Residence site		
Urban	188	2.41 (1.11, 5.24)
Rural	76	2.57 (0.79, 8.36)
p-interaction		0.696
Residence site		
Distant from agrarian cultivation	174	2.11 (0.96, 4.63)
Near agrarian cultivation	80	4.84 (1.24, 18.90)
p-interaction		0.436
Residence site		
Distant from a garage	208	1.69 (0.79, 3.63)
Near a garage	44	4.28 (1.19, 15.45)
p-interaction		0.060

MN, micronuclei; BN, bi-nucleated T lymphocytes; MN1000BN, MN per 1000 bi-nucleated T lymphocytes; PCBs, polychlorinated biphenyls

6. GRAPHS

GRAPH 1: Adjusted associations of first trimester maternal serum POP levels with micronuclei frequency at 4 years of age, mother-child cohort, Crete, Greece.



MN, micronuclei, BN, bi-nucleated T lymphocytes; MN1000BN, MN per 1000 bi-nucleated T lymphocytes; DDE tertiles: low (189.85-1342.44 pg/ml), middle (1360.21-2734.08 pg/ml), and high (2738.37-23175.4 pg/ml), HCB tertiles: low (19.52-68.63 pg/ml), middle (68.71-101.19 pg/ml), and high (102.49-1330.51 pg/ml), PCBs tertiles: low (66.23-247.45 pg/ml), middle (247.62-412.19 pg/ml), and high (413.37-1988.38 pg/ml), Dioxin like PCBs tertiles: low (6-18.95 pg/ml), middle (19.13-30.51 pg/ml), and high (30.62-190.68 pg/ml), non Dioxin like PCBs tertiles: low (59.78-226.94 pg/ml), middle (227.28-383.05 pg/ml), and high (386.83-1927.95 pg/ml).

Adjusted for child's sex (boy, girl), child's exposure to passive smoking (yes, no), maternal age (continuous variables), smoking during early pregnancy (yes, no), breastfeeding duration (months), pre-pregnancy BMI (kilograms per meter squared), parity (primiparous, multiparous), maternal educational level [low (≤ 9 years of mandatory schooling), medium (> 9 years of schooling up to attending postsecondary school education), high (attending university or having a university/technical college degree)] and maternal serum triglycerides and cholesterol.

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