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Dioxin-like activity in blood from women with breast cancer and their age-matched controls

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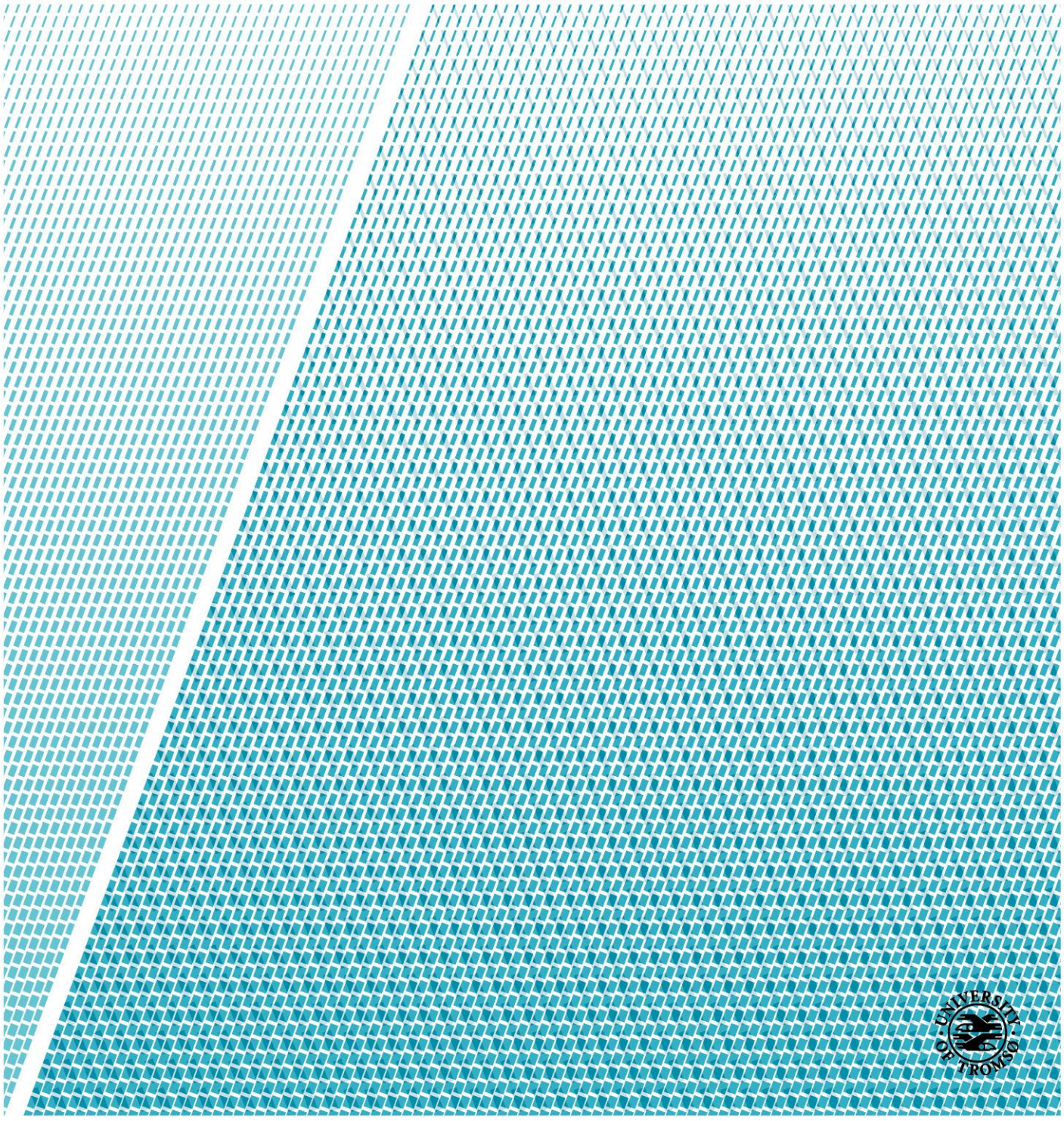
MED-3950 Master's thesis in Medical Profession, kull 12



Preface

I have been interested in what kind of effects all chemicals humans are producing and releasing into the environment may have on the environment, wildlife and humans. Therefore, I asked Torkjel Sandanger, which I had seen on the news on TV talking about environmentally damaging chemicals in baking paper and food wrapping on behalf of the Department of community medicine, University of Tromsø, if he had a project I could join. He assigned me this project to write under the supervision of Charlotta Rylander. The data in the study comes from the nationwide survey The Norwegian Women and Cancer (NOWAC) conducted by the Department of Community Medicine at the University of Tromsø and has long been waiting to be examined if there are any associations between dioxin-like activity in blood serum and risk of breast cancer.

I must give a great thank you to Charlotta, who has helped me enormously with learning all the basics necessary to perform the statistical analyzes and guided me in how to write the thesis and keeping a time schedule. Also thank you to Torkjel, who was very helpful in getting me involved in the project and has given great advice and guidance as well.

Lise Custers Nedrebø

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Summary

Breast cancer is the most common cancer in women today, and although many risk factors are known, these cannot fully explain the vast increase in breast cancer incidences the last decades. Persistent organic pollutants have been indicated as potential carcinogenic compounds and it is therefore interesting to see if they can be a factor in the development of the disease. Here, we assessed the association between dioxin-like activity in blood and the risk of breast cancer. 290 women in the ages 41-55 years who participated in the nationwide survey The Norwegian Women and Cancer study (NOWAC) answered questionnaires and donated blood samples in the years 1999 to 2006. 98 of these were diagnosed with breast cancer. The blood samples were analyzed using an AhR-responsive reporter gene bioassay, CALUX, to measure the dioxin-like activity. We found no significant association between the dioxin-like activity and the risk of breast cancer. These findings are similar to what the majority of other studies have found. However, limitations in the sample size and possibly the time of blood sampling are important factors when evaluating the results, and more research is needed in the area.

1. Introduction/background

Breast cancer is the most common cancer type in women today and the most important reason to death in women under 65 years(1). The last 60 years, there has been a doubling in the incidence of breast cancer in Norway (2). Many risk factors have been established in the pathogenesis of breast cancer, one of them being that the population becomes increasingly older. However, the established risk factors do not fully explain the great increase in breast cancer incidence (3). This has made researchers look to environmental pollutants, in particular dioxins and dioxin-like compounds(DLCs), to assess any associations with breast cancer. Many studies have been conducted, but mostly leading to inconclusive results. Animal studies have shown significant associations between dioxins and DLCs and different cancers, but it has been difficult to assess the same associations in humans.

1.1 Breast cancer

Breast cancer is the most common cancer among women today; 22 % of all female cancer cases in Norway is breast cancer (2). Over the last decades the incidence has increased substantially; the age adjusted incidence have gone up from 60.6 per 100 000 in 1958-60 to 123.3 per 100 000 in 2011-15 (2). Breast cancer is currently the most important cause of death in women under 65 years, thus even more important than cardiovascular disease and accidents (2). The 5-year survival rate has in the last 35 years increased from 69.8% to 89.0%, although the prognosis depends largely on the stadium of the tumour at diagnosis (2). In 2013, the 5-year survival was 98.9 % for tumours located only to the breast, while it was 25.1 % if there were metastases at the time of diagnosis(1). Established risk factors for developing breast cancer are higher age(4), hormone replacement treatment during and after menopause(5), early menarche(6), late menopause(6), having no children(7), having the first child at an older age (35 years)(7), and having a genetic predisposition or having a mother or sister with breast cancer (8). Breastfeeding however, lowers the risk (6). Changes in people's life style are also important contributors; smoking, alcohol consumption, intake of fatty foods and a decreased activity level are considered factors that increase the risk of breast cancer, as well as postmenopausal obesity (7). There are also geographical variations in breast cancer, where the incidence and mortality is much

greater in industrialized western countries than in the Far East. It is shown that first or second-generation migrants from Japan to Hawaii acquire the breast cancer rates in the host country, indicating that environmental factors play a more important role than genetic factors (7). Even though all of these risk factors are known, they only explain 40-50 % of the breast cancer incidences (3). Mammography, a breast cancer screening programme for women of 50-69 years, detects more cancers in earlier stages and contributes to more tumours being detected, possibly tumours that would never have become cancers (9). However, this increased detection of possible benign breast tumours does not fully explain the increase in incidences. Nor does the increasing age of the population, change in lifestyle or the other established risk factors. This, and the fact that animal studies have indicated that several persistent organic pollutants are carcinogenic, has made researches look to environmental pollutants to assess any associations with breast cancer.

1.2 Dioxins and DLCs

1.2.1 Dioxins – a specific group of persistent organic pollutants(POPs)

POPs are organic compounds that persist in the environment, accumulate in organisms and exert toxic effects. They are semi-volatile compounds, enabling them to travel long distances in the air (10). Common for all POPs is that they are slowly metabolized or degraded and thus being persistent (11). Many POPs have been identified. A group of compounds belonging to POPs are polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), commonly called dioxins. They are planar tricyclic aromatic compounds, the PCDDs contain a dibenzo-1,4-dioxin molecule and the PCDFs a a dibenzofuran molecule, each with 1 to 8 chlorine atoms attached at different positions (Figure 1) (12). There are in total 210 different dioxin congeners of the dioxins, 75 PCDDs and 135 PCDFs. The 2,3,7,8–chlorosubstituted congeners bioaccumulate the most and are the most toxic congeners, and these make out a total

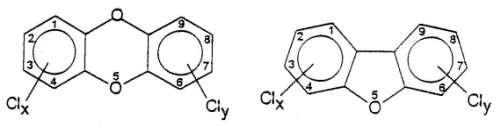


Figure 1: The general structures of PCDDs (left) and PCDFs (right) (13)

of 17 congeners(13). The most toxic dioxin is the 2,3,7,8-tetrachlorodibenzo-p-dioxins, or TCDD (13).

1.2.2 DLCs

Several chemicals with dioxin-like activity exist, but the most common DLCs are polychlorinated biphenyls (PCBs). Biphenyls are molecules consisting of two benzene rings connected with a C-C-bond, and in polychlorinated biphenyls, one to ten chlorine atoms are attached to the rings at different positions (Figure 2) (13). A total theoretical number of 209 possible PCB congeners exist, of these, 13 have chlorine atoms only in meta- and para-positions, making them similar to dioxins in structure, and gives them their dioxin-like toxicity (14).

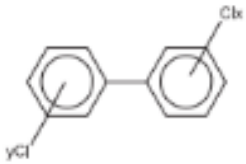


Figure 2: PCB molecule (13).

1.2.3 Physiochemical properties

The properties of the different congeners of dioxins and DLCs vary, but in general they are highly stable both thermally and chemically and can persist in the environment for decades(15). It can take from 7 to 12 years to remove half of the TCDD from the human body (16). Mostly, dioxins and DLCs have high boiling points and low vapour pressures(13). They are lipophilic and therefore little soluble in water, but resolve well in oils, organic solvents and fats (13). In the environment, the congeners are mostly found as mixtures and not as single compounds (13).

1.2.4 Sources of dioxins and DLCs

Dioxins are unwanted byproducts from chemical processes involving chlorine and combustion and have never been produced deliberately or have any known use(11). Examples of industries producing dioxins as byproducts are paper manufacturing, waste incineration, smelting and the manufacturing of certain types of pesticides and herbicides. Natural sources are forest fires and volcano eruptions(17). Dioxins were also a by-product of Agent Orange, a blend of two herbicides that the US Army sprayed over the forests in Vietnam as a defoliant agent (18). It removed trees and dense tropical foliage that the enemy used as coverage during the Vietnam War in 1962 to 1971 (18). The production of PCBs started in 1929 for use as insulating agents in transformer oils and capacitators, as heat transfer agents, as additives in paints, papers and plastics, and in sealants for constructing(13). It was sold under names like Aroclor,

Fenclor and NoFlamol (14). The production of PCBs was banned in the US in 1979 by the US Environmental Protection Agency, and the disposal of PCBs were put under regulations(19). Both dioxins and dioxin-like PCBs have been classified as group 1 carcinogens (20). In 2001, The Stockholm Convention, a global treaty to protect the human health and the environment from POPs, was put into force and signed by 152 countries worldwide (21). The aim was to eliminate dangerous POPs and support the transition to other safer alternatives. Initially, 12 POPs were targeted in the convention, including some PCBs and dioxins, and today many more POPs have been added(21). The convention requires the members to eliminate or reduce the release of POPs into the environment(21). Even though many countries have signed the treaty, many of them has yet to ratify them, including the US(22, 23).

1.2.5 Transfer of dioxins and DLCs in nature

Dioxins and DLCs are highly stable both thermally and chemically, which is the reason why they are ubiquitous in the environment today. Degradation mechanisms are slow, and intentional degradation requires either high temperature or catalysts(24). Dioxins and DLCs may get degraded by sunlight, but is sheltered if attached to particles(25). From production, use and disposal, they end up in soil and water where they are mainly found bound to particulates because of the low water-solubility(13). From soil and water, they reach rivers and oceans, where currents transport them to the Polar Regions(26). However, the main transport route is through the air (13). The dioxins and DLCs are volatile and have high affinity for air particulates, which enables the compounds to spread through the atmosphere. Gravity, precipitation and wind currents move the dioxins and DLCs to the ground where it is deposited in soil and water. Wind currents transport them to Polar Regions where they get cooled down, condensate and get deposited (26). The transport mechanisms are illustrated in Figure 3.

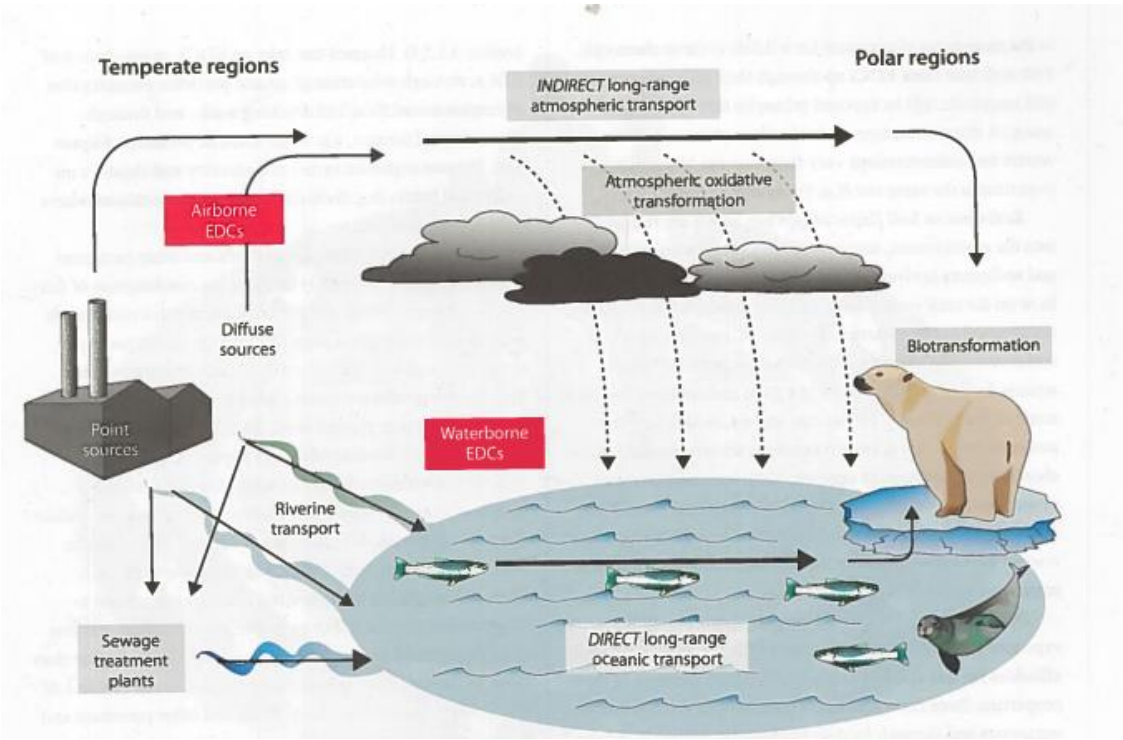


Figure 3: Transport mechanisms of persistent organic pollutants and bio magnification in organisms (10).

The lipophilic character of the dioxins and DLCs makes them easily absorbed into organisms (13). They get incorporated in smaller organisms, such as plant plankton and animal plankton which gets eaten by bigger organisms, which again are eaten by bigger organisms, and as the dioxins and DLCs get stored in the fat, it gets accumulated through the trophic levels. The highest concentrations are found in the animals highest in the food chain, e. g big fish, sea gulls, polar bears, polar foxes, whales and seals (Figure 3) (10).

1.2.6 Human exposure routes to dioxins and DLCs

Previously, occupational exposure was a major route of dioxins and DLCs to humans (27). Studies have shown that workers in factories producing chemicals with TCDD as byproducts, such as herbicides, pesticides and trichloropropane, had much higher levels of dioxins than the background controls(27). Accidents in these factories would also lead to high TCDD levels in the workers doing the cleanups, as well as work involving clean-up of PCB waste-sites, PCB disposal activities or work with old products containing PCBs, such as x-ray machines, welding equipment, refrigerators, televisions and microwaves made before the 1980s (28). Another important source for PCB exposure is living close-by incinerators or other PCB-disposal facilities (28). Renovation

work involving plaster, paint, and caulk, that contain PCBs also puts people at risk for PCB exposure. In Norway there has been little production of PCBs or other major POPs, although occupational exposure has been an issue since there has for example been production of windows with PCBs and furniture with BFRs(29). However, exposure is most likely to be from a combination of earlier import and usage, long-range transport and food(30). PCBs and dioxins can get into the human body through breathing and skin contact, but the main entrance way is through ingestion(31). Today, the major sources for dioxins and PCBs for the general population are foods such as fish, dairy products, eggs and meat(31). The greatest contributor of dioxins and DLCs in the Norwegian diet is fatty fish like mackerel, herring, salmon, halibut and trout(32). Seagull eggs, cod liver, big halibuts and the brown meat in crabs contain very high levels of PCBs and the Norwegian Food Safety Authority recommends limiting the intake of these foods, especially for women in childbearing age(32). During pregnancy, dioxins and DLCs are transferred to the fetus via the placenta(10), and they can also be transferred to the newborn through breastmilk (33). Since breastmilk may be the only or predominant food source to the infant, the infant receives high doses of the chemical pollutants (34). However, the amount of PCDDs in human milk decreases as the breastfeeding period increases, and also decreases with successive breastfed children (35). The Norwegian Scientific Committee for Food Safety (VKM) has set a Tolerably Weekly Intake (TWI) of dioxins and DLCs to avoid being at risk for damaging effects from them(36). TWI is the weekly amount of a substance that can be consumed safely throughout a lifetime without risking adverse health effects from environmental chemicals in the substances(36). The TWI in Norway is 14 pg TEQ/kg bodyweight/week, so for example, an intake of 300-450 g of fish per week, included 200 g of fatty fish, is well below the TWI (36).

1.2.7 Trends of levels of dioxins and DLCs in humans

Since the PCBs and dioxins are lipophilic, they get stored in adipose tissue. An equilibrium is established between fatty tissue and the blood, so that the blood serum/plasma gives a good indication of the levels of pollutants in the body(37). However, the concentration in the blood and adipose tissue in 2017 does not reflect the concentration in the blood and adipose

tissue 30 years before, seeing as the levels of POPs in both the environment and the humans had a major drop following the restrictions made on the production and use of the POPs during the 70s and 80s (38). Biomonitoring studies of POPs and other environmental pollutants have been conducted to assess human health risks from exposure to environmental contaminants. The Arctic Monitoring and Assessment Programme (AMAP), a group working under the Arctic Council, do biomonitoring activities in the eight arctic countries; Russia, Canada, Greenland, Norway, Sweden, Iceland, Finland and the Faroe Islands. Here they evaluate the conditions of the Arctic ecosystems, identify possible causes for changing conditions, detect emerging problems and recommend required actions to reduce risks to Arctic ecosystems(39). In 2015 they reported that the levels of most POPs in humans had declined since 1979, as seen in measurements done in Norwegian men from 1979 to 2009 showing a median reduction of 69 % of POP levels in blood serum, including PCBs (excluding dioxins) (38). This is consistent with the reduction in emissions following international actions to reduce or eliminate production of POPs in the same time period (38). Pregnant women in Nunavik, Greenland donated blood samples in 1992 to 2013, and overall, levels of POPs in their blood declined by an average of 80 %. These reductions are thought to come from international actions eliminating the production and emission of POPs, but also from people eating more store-bought food and less traditional food(marine mammals and fish), because of governmental recommendations and from cultural changes(39). The trend in dioxin levels has not been as extensively studied as for the PCBs, but studies show that dioxin levels has followed the same pattern as for PCBs (40, 41). Aylward and Hays (41) surveyed literature reporting TCDD levels in samples from the general population in the US, Canada, Germany and France. They found a steady and substantial decline in TCDD levels from 1979 to 2000 and concluded with an exposure reduction of 95 %. Their tentative reasons for these declinations included reductions in open burning practices at municipal landfills, homes and apartment buildings; reduction in TCDD levels in herbicides used in the United States and subsequent suspension of the use of these herbicides; reductions in incinerator emissions due to new regulations and equipment; and lifestyle changes such as changes in dietary patterns (41). In Norway, the emission of dioxins has been reduced with approximately 70 % from 1995 to 2013 according to the Norwegian Environmental Agency(42). Measures to reduce the dioxin load on the environment are stricter standards on emission for waste burning industries and other industries. Heating of

houses has also been improved in regard to emissions of dioxins, and the goal is to have ceased emissions of dioxins in Norway within 2020(42).

Because of interventions such as the Stockholm Convention, a considerable decline for many POPs has been observed. However, the number of organic and inorganic substances that are being introduced to the global market are substantial, and over 100 000 substances are available in the commercial market(30). These have not yet been identified as toxicants, but could be potential hazards to the environment and humans (30).

1.3 Endocrine disruption and health effects of dioxins/DLCs

Animal experiments and accidents as well as occupational exposure show that dioxins and DLCs cause negative health effects. The question is however at what concentrations these compounds cause effects in humans from the general population that are exposed to low concentrations over the whole lifespan. Dioxins and DLCs function as endocrine disruptors, which means they interfere with normal hormone action and can in principle affect any hormonal system containing receptors the chemicals can act through (39). In the 2002 report *Global Assessment of the State-of-the-Science of Endocrine Disruptors* by the International Programme on Chemical Safety (IPCS), endocrine disruptors were defined as *"an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or (sub)populations* (10). Endocrine disruptors can act throughout life just as hormones do, impacting the same pathways as the hormones. If the endocrine disruptors are present during development, programming of cell and tissue development will be affected and lead to permanent effects(39). Endocrine disruption early in life will remain throughout life and the tissue will have a different predisposition for disease later in life compared to a non-exposed subject(39). For example will exposure to the endocrine disruptor bisphenol A to a fetal mice predispose the mouse to prostate-cancer if exposed to low levels of estrogen later in life(39). If present later in life, endocrine disruptors can exert different effects, which may be transient(39). An example of the endocrine disruptive character of dioxins and DLCs is the antiestrogenic effects shown in many studies

where TCDD have had toxic effects on ovulation, uterine function, pregnancy, endometriosis and embryonic development (43). Furthermore, animal studies have shown that dioxins and DLCs can suppress ovarian follicle growth stimulated by gonadotropins, inhibit the preovulatory LH-surge and alter the hormone expression through the menstrual cycle (43).

Dioxins and DLCs can exert many other adverse health effects as well. Acute health effects have been observed in workers exposed to dioxins, in the form of rashes called chloracne(13). This was also seen in the candidate for the presidential election in Ukraine in 2004, Victor Yushchenko, after being intoxicated with large amounts of TCDD (44). Established chronic effects from exposure to TCDD are dermal toxicity, immunotoxicity, reproductive effects, teratogenicity, endocrine disruption and carcinogenicity (34). The dioxins and DLCs transferred through the placenta can give disrupted neurodevelopment, and if present in breastmilk they can lead to transiently damaged liver in the newborn, as well as reduced IQ and altered behavior(45). Examples of cancers that may be associated with dioxins and DLCs are liver, thyroid, lung, endometrial, breast and testicular cancer(46). Dioxins and dioxin-like PCBs are the only POPs that have been classified as Carcinogenic Chemicals to Humans by the International Agency for Research on Cancer (47).

Animal studies have also shown a range of different adverse health effects; exposure of low TCDD levels to monkeys during perinatal development led to learning disabilities in the monkeys (48). Rodents exposed to dioxins and DLCs developed endometriosis (49), liver damages (50), reduced reproductive potential of females, permanently reduced sperm count in male progeny and urogenital malformations in both sexes (51). Immunological effects have also been seen, such as thymus size reduction, increased susceptibility to infectious diseases and suppression of immune functions (52). Neurodevelopmental and other developmental disruptions is also established effects of exposure to dioxins and DLCs, as seen in developing fish embryos getting craniofacial malformations and neural damage after exposure (53). Lethality is also an end-point of dioxins and DLCs exposure to animals (54). However, in this study, the endocrine disruptive properties of the dioxins and DLCs are the most interesting and will be further explained later.

1.3.1 Endocrine disruption

To understand the endocrine disruptors, it is important to understand how the hormonal, or endocrine, system works. The endocrine system consists of organs in the body which produces and releases chemical substances, hormones, into the blood, where it travels to other tissues and organs and exerts their effects(10). An example is the hormone insulin produced in the pancreas, which induces glucose uptake in various cells of the body when released into the blood stream (10). The effects on the cells depend on the cell having the right receptor for the hormone. Protein and amine hormones can travel freely in the blood, but cannot diffuse into cells, and therefore act on receptors outside the cell that forwards the message(10). Some hormones, such as steroids, are transported on proteins in the blood and can passively diffuse into cells where they connect to intracellular receptors and exert direct effects on changes in the cell. The steroid hormones also have receptors on the cell membrane outside the cell, and these receptors are considered to be important in how exogenous substances influences the cells(10).

Because the receptors have high affinity for the hormones, the hormones can act at very low concentrations and even small amounts of a hormone can initiate important effects on the cells. The hormones produce a sigmoidal dose-response curve, where small increases in a hormone at low concentrations gives a large increase in effect as seen in Figure 4. This is highly important when considering the effects of endocrine disrupting chemicals, even at small concentrations. The effect also depends on the amounts of receptors on the specific cells. A cell with more receptors will have a dose-response curve shifted to the left and vice versa(10).

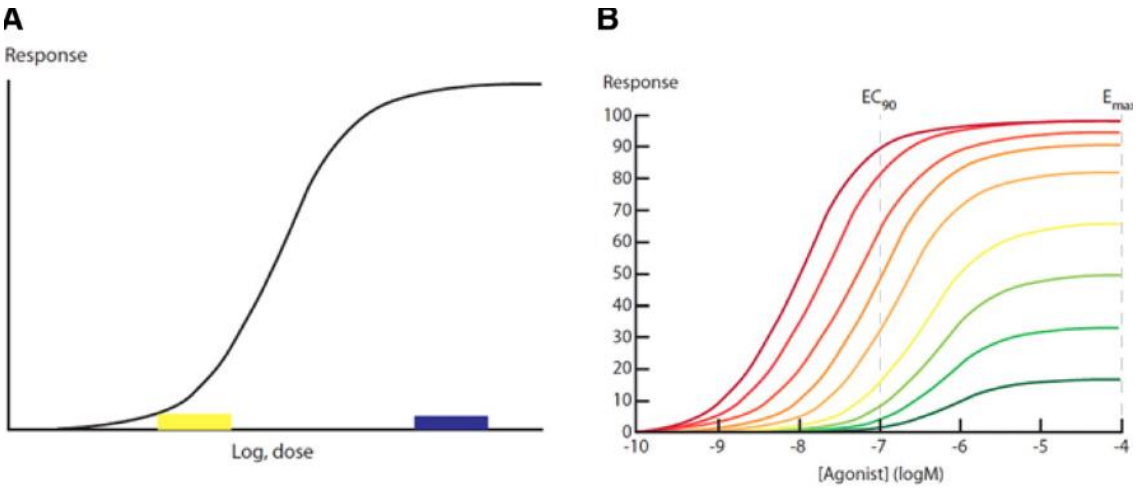


Figure 4: (A) dose-response curve for hormones. (B) more receptors on a cell will shift the curve to the left and lead to biological effects at lower concentrations(10).

However, dose-responses can be more complex because the receptors are activated by the hormones in a non-linear fashion, creating a non-monotonic dose-response curve(10). Specific hormones can exert maximal effect in the highest and lowest concentrations and create a U-shaped dose-response curve. If the maximal effect comes at intermediate levels, the curve will be an inverted U(10). The inverted U-shape could be an effect of more than one receptor being involved, where one receptor is inhibited and one is activated by the same hormone, so that at intermediate levels, there will be maximum activity of both receptors combined(10). The non-monotonic curve can also be a result of receptor down-regulation. Receptors are degraded when the hormones act at high concentrations(10). In addition, hormones can exert a toxic effect at high doses, and lead to cell death, which also gives a non-monotonic dose-response. Lastly, the affinity of the receptors can change at different hormone concentrations, and at high concentrations hormones can bind to other receptors as well as their specific receptors, and lead to an increased effect(10).

Generally, there are two ways for a chemical to act as an endocrine disruptor; directly acting on a hormone receptor, or indirectly through affecting hormone delivery(10). When designing studies to link human exposures to specific outcomes, the chemical exposure needs to be measured at the developmental time-point appropriate for the specific outcome that is being assessed(10). Example of endocrine disruptors are PCBs, dioxins, DDTs, chlordane and hexachlorobenzene(10). The endocrine disrupting properties of dioxins and DLCs are explained later in the text.

1.3.2 Estrogen and estrogen receptor

Estrogen and estrogen receptor(ER) is thought to play an important role in the development of breast cancer, although the mechanisms of how estrogens increase the risk of breast cancer are not completely clear. Most human breast cancers are positive for ER, where chemicals with estrogenic activity can stimulate and chemicals with anti-estrogenic activity can inhibit growth(55). The estrogen and estrogen receptor is considered to be important for the toxic effects of dioxins and DLCS (56). The female sex hormone estrogen is important in the development, maturation and functions of female reproductive organs and breasts, and plays an important role in the development of secondary female characteristics. It has also an effect on fat tissue and the skeletal, vascular and neural system(57). Estrogen is a family of hormones circulating in the blood bound to the protein SSBG (sex steroid binding globuline) and most of their actions on cells are mediated through Estrogen Receptors(ER) within and outside the cell. The estrogen receptor is known in two forms, ERα and ERβ, where ERα was discovered first and is the most extensively studied receptor(58). The ER is an important component in cell cycle progression and the main effect comes from acting on the receptors within the cell, where binding will lead to changes in DNA-transcription. This is called genomic effects. If the hormone binds to receptors on the cell membrane, it will lead to quick responses in the cell, e.g activation of MAP kinases and PI3-kinases(57). Activation of PI3-kinases leads to inhibition of apoptosis(59), activation of MAP-kinases leads to cell proliferation and cell survival(60). These are non-genomic effects. The ER have low specificity and can bind a range of different estrogen types and also other chemicals with similar structures (57). Activation of the ER is thought to happen through two main pathways; the “classical” pathway induces ER through an agonist, e. g estradiol (E2), and leads to direct interaction between ER and DNA and subsequent transcription. In the non-classical way, agonists induce the ER to interact with other proteins which then bind to DNA and activate transcription(55). As previously noted, many factors related to estrogen production is associated with breast cancer, i.e early menarche, late menopause, obesity and use of hormone replacement therapy. Santen et al.(61) proposed two possible mechanisms for the increased risk; a) breast cell proliferation with simultaneous enhanced rate of

mutations stimulated by ER and b) estradiol metabolized into genotoxic metabolites leading to increase in DNA mutations (61).

1.3.3 The Aryl Hydrocarbon receptor

The Aryl hydrocarbon Receptor(AhR) is a ligand-dependent transcription factor part of the basic helix-loop-helix(bHLH) family of gene regulatory proteins. The receptor has been extensively studied, and it is considered to be the main pathway for the toxic effects of dioxins and DLCs as well as other halogenated aromatic hydrocarbons(62). These lead to AhR-dependent toxic and biological effects, such as chloracne, cancer and immune suppression in humans and hydronephrosis and cleft-palate in mouse embryos(63). Other exogenous chemicals have also been identified as AhR-agonists, (e.g. 2-(1'H-indole-3-carbonyl)-thiazole-4-carboxyl acid methyl ester), but does not lead to the toxic effects seen when dioxins and DLCs activates AhR(64). Although no endogenous ligands for the AhR have been identified yet, bHLH factors have in general critical roles for embryonic development, and so it is likely that the AhR has a physiological function in development. Studies with mice with knockout AhR genes were viable and fertile, but developed hepatic defects, indicating a role of AhR in normal hepatic development(27). TCDD is considered the most potent activator of the AhR of the halogenated hydrocarbons and induces the trancription of CYP1A-enzymes(65). CYP1A is a group of enzymes comprising of CYP1A1 and CYP1A2 and is part of the P450 superfamily metabolizing xenobiotics (chemicals that are foreign to the body), like drugs or environmental pollutions. CYP1A enzymes both activates and detoxify several environmental carcinogens, such as polycyclic aromatic hydrocarbons found in combustion products from cigarette smoke and incineration(66). CYP1A1 are mainly extra-hepatic, while CYP1A2 is mainly found in the liver, and both are regulated by the AhR(62). The constitutive expression of CYP1A is relatively low in most tissues and cells, but treatment with dioxins and DLCs leads to accumulation of AhR in the nucleus and subsquent CYP1A-induction(27). When a ligand has diffused into the cytosol of the cell, it binds to the AhR, which is bound to two heat shock proteins, leading to a conformational change and translocation into the nucleus. Here the proteins dissociates and the AhR binds to a nuclear protein called ARNT (AhR nuclear

translocator). AhR-Arnt binds to the dioxin or xenobiotic response elements (DRE/XRE) on the DNA and induce transcription of the CYP1 gene(62). This is illustrated in Figure 5.

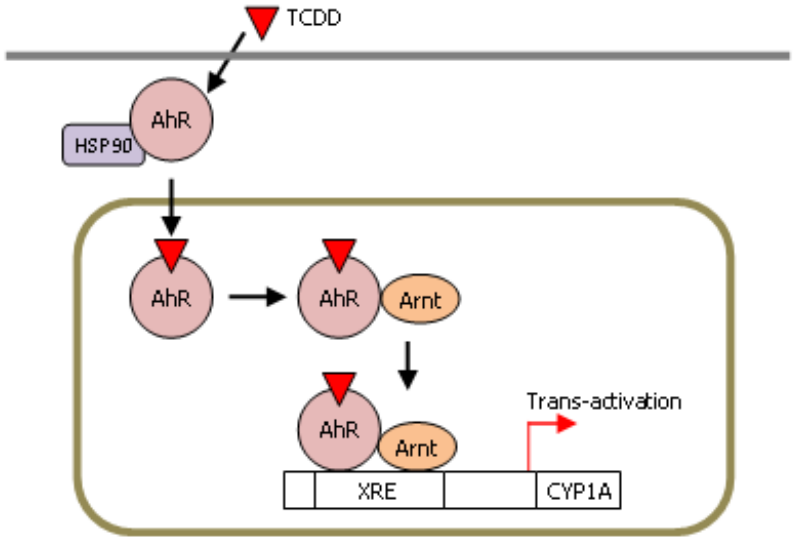


Figure 5: Activation of AhR leading to DNA transcription and production of the enzyme CYP1A1 (67).

The dioxin- or DLC-induced CYP1A enzymes' activation of carcinogenic PAHs and heterocyclic amines/amides lead to formation of reactive intermediates which can cause a wide range of toxicities and cancer(62). Examples are the reactive oxygen species(ROS) H_2O_2, superoxide and hydroxyl radicals(62, 68), which are important in promotion of tumor development and progression(69), and catechol estrogen, which exerts cytotoxic DNA-damage(70). The majority of the toxic effects of dioxins and DLCs are observed weeks after exposure and the adverse effects most likely arise from continous and inappropriate expression of specific genes resulting in the delayed toxic response(65). The ligand-activated AhR pathway can also alter expression of other endocrine receptors and ligands, such as the Estrogen Receptor α in AhR-ER crosstalk, which has been observed in human breast cancer cells(64). This crosstalk is induced by dioxins and DLCs and inhibits E2-induced responses through the AhR via several mechanisms (71). Firstly, the activation of CYP1A1 leads to the metabolism of estradiol, depleting intracellular levels of the hormone and yielding hydroxy metabolites, that are further oxidized into quinone and semi-quinone forms which can

alter DNA(64). If this continues over time, the mutations accumulate and can lead to neoplastic transformations(58). Secondly, dioxins and DLCs activate proteasomes which degrade ERα. This inhibits effects of the ERα and leads to ubiquitination of ERα(64), an important regulation process of the cell cycle(64). Research is being done to find AhR-antagonistic drugs that can target the Ah-receptor and inhibit its activity; 6-MCDF is an identified selective AhR modulator (SAhRM) inhibiting TCDD-induced CYP1A1(64).

1.4 Epidemiologic studies on associations between dioxins, DLCs and cancer

10th of July 1976, an explosion in a chemical manufacture plant in Seveso, Italy resulted in the release of high levels of dioxins(72). A retrospective study was initiated in 1996 where 981 women in the age of 0 to 40 years and living close to the explosion area in 1976 were included to assess the associations between TCDD and breast cancer (72). Blood serum had been sampled short time after the explosion (1976-1981), archived and was analysed for TCDD using high-resolution mass spectrometry (HRMS). For some of the women (3 %) the blood samples were of insufficient volumes and new samples were collected in 1996-97 (72). For the women giving blood samples after 1976, the TCDD levels were back-extrapolated to 1976 using a 9-year half-life and first-order kinetic model (72). The 15 participants developing breast cancer had TCDD level ranges from 13 to 1960 parts per trillion (72). A significant association of TCDD with breast cancer was found with a hazard ratio of 2.1 (95% CI 1.0-4.6) (72). 833 women participated in the follow-up study in 2008, of these, 66 had now been diagnosed with cancer, 33 of which with breast cancer(73). They then concluded with serum TCDD being significantly associated with all cancers combined (HR 1.80, 95% CI 1.29-2.52), but not significantly associated with breast cancer (HR 1.44, 95% CI 0.89-2.33)(73). Limitations in both studies were few breast cancer cases and back-extrapolation of serum-TCDD for many of the samples (73).

Zhang et al(74) conducted a meta-analysis on the association between PCBs and breast cancer risk through november 2014. They included 25 studies, involving a total of 6088 cases and 6778 controls from eight countries and found a positive association between

dioxin-like PCBs and the risk of breast cancer, as well as between CYP1a and CYP2b inducing PCBs and breast cancer. Recio-Vega et. al(75) measured 20 PCB congeners, including dioxin-likes, in the blood serum(year of blood sampling not specified) of 140 Mexican women, of which 70 were diagnosed with breast cancer, using gas chromatography(GC)–electron capture detection. A positive association was seen for PCBs and breast cancer, although only for non-dioxin-like PCBs. An increased odds ratio was seen for the dioxin-like PCBs, but not significant.

However, many of the epidemiological studies assessing the relationship between dioxins, DLCs and breast cancer have found no associations between dioxin, DLCs and breast cancer(76-81). Some researchers are of the opinion that IARC's classification of dioxins and DLCs as Class 1 Carcinogens are based on too weak evidence material (76, 82). Boffetta et al(76) did a critical review of the epidemiologic studies done on exposure to TCDD and cancer risk between 1997 and 2010 and concluded that dioxins and PCBs should be considered less toxic and carcinogenic then the IARC had determined in 1997. Zheng et al (83) investigated the relationship between DDE and PCBs and breast cancer by measuring the concentrations in blood serum sampled in 1995-1997 from women in Tolland County or New Haven county in the US. They used GC in analysing the samples(detection method not specified). Their 475 cases were incident breast cancer cases, and the 502 controls were randomly selected from residents of Tolland county or patients at Yale-New Haven Hospital with newly diagnosed benign breast disease or normal tissue (83). They found no major increase in breast cancer risk associated with any congeners of PCB, also not for the dioxin-like PCBs they tested for, although they only included two DL-PCBs (83, 84). Danjou et al (77) estimated dietary exposure among 63,830 French women followed from 1993 to 2008 in the E3N cohort study. They based the exposure on questionnaires about diet history and food dioxin contamination data from a French national monitoring program. They found no significant association between breast cancer risk and dioxin exposure(77). Reynolds et al (79) evaluated the association between breast cancer and dioxins through measuring dioxins in breast adipose tissue in 79 incident breast cancer cases and 52 controls, diagnosed with benign breast disease in the mid-1990s. They measured the samples by high resolution GC-MS and found no significant associations.

Morgan et al(85) did a case-control study of breast cancer using 403 matched pairs from the National Health and Nutrition Examination Survey (NHANES), conducted in 1999-2004. Blood serum samples were examined for POPs, including PCBs(analysis method of measuring PCBs not specified). They found a positive association between breast cancer and the non-dioxin-like PCB 138, but not for total-PCBs or dioxin-like PCBs. Limitations in the study was a small study population and the use of cross-sectional self-reported cancer status data. Ward et al(81) investigated the associations between organochlorines and breast cancer in 300 Norwegian women using blood samples collected in 1973 to 1999 stored in the Janus Serum Bank in Norway. They examined organochlorines, including dioxins and PCBs, using high resolution GC-MS and found no associations on elevated breast cancer risk with these chemicals. However, they did not include the most toxic dioxin, TCDD, in their results because of too low detection rates. Xu et al(78) did a meta-analysis on the associations between dioxin and cancer incidence and mortality in July 2015. Within this study they conducted subgroup analysis according to cancer subtypes, including breast cancer. They reviewed 12 different studies assessing the exposure to dioxins, and in total they had a number of 3768 cases and found no significant risk for breast cancer(78). Mouly and Toms evaluated 14 case-control studies and one cohort from 2006-2014 to summarize and integrate the risk of breast cancer following environmental exposure to POPs, other than DDT(86). 8 of the studies considered the effect of PCB exposure, both non-dioxin like and dioxin-like, measuring PCB in serum or plasma, one study measuring PCB in adipose tissue. They found inconsistent and inadequate evidence to conclude with any certain associations with breast cancer (86). They pointed out the weakness of many of the studies having examined the exposure to POPs after the time of diagnosis, possibly overlooking exposure at critical vulnerable windows in the females' lives where the breast could be more susceptible to endocrine disruption, such as in-utero, puberty, pregnancy or postpartum. Also, the studies had focused more on individual chemical compounds, possibly missing effects from chemical pollutants combined (86).

1.5 Quantifying exposure to dioxins and DLCs

1.5.1 Assessing dioxins and DLCs in humans

It is a challenge to correctly assess the concentrations of all dioxins and DLCs in blood, since concentrations are low and there is a large number of relevant compounds. Furthermore, one snapshot might not be a good estimate of lifetime exposure or earlier exposure at sensitive time windows like the in-utero period and early life. At the same time individuals may not know that they are exposed, nor to what degree, so asking questions concerning exposure is of little value. A way of measuring current or former exposure is to ask questions about profession, place and length of residency and assessing other activities, and comparing with historical data or area measurements of the level of pollutants. However, grouping of work titles, area measurements and indirect exposure can lead to the exposure of an individual being assessed incorrectly, because the actual exposure degree depends on the individual's experience and activities(87). An easier way to assess an individual's exposure is using biomarkers. Tissues or blood can be used to extract chemical pollutants and thereby measure the internal dose in humans(87). An important factor to take into account when assessing the levels of dioxins and DLCs in individual women is that these compounds are transferred through the placenta and through breastfeeding, thus concentrations are lower in a woman after pregnancy and a period of breastfeeding(33, 88). Therefore, women having had many children and breastfed for many months may have lower levels of dioxins and DLCs than women not having any children, even though they have been exposed for the same dioxin and DLC levels(33). Age is also a highly relevant factor to be taken into account when comparing dioxin and DLC levels in individuals, as an older person most likely will have accumulated higher concentrations through a longer lifetime(4).

1.5.2 GC-MS and CALUX bioassay

The golden standard for analyzing dioxins and DLCs is the gas chromatography combined with mass spectrometry (GC-MS). Gas chromatography separates the chemical compounds of a mixture(89), and the mass spectrometry determines the mass of the separated compounds(90). Using these two methods in combination gives a high certainty of the results(90). In biological samples, isotope-dilution high-

resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) is a very sensitive and specific technique to quantify the concentration of pollutants in the samples(91). The concentrations of the different dioxins and DLCs are weighted in regard to their TEF-values and then added up to get a TEQ-value (TEF and TEQ are explained in the next paragraph)(92). This is however an expensive and time-consuming technique, and requires large volumes of samples(92). Therefore, other techniques have been developed to analyze dioxins and DLCs, and in this study an AhR-dependent recombinant bioassay has been used to estimate the total dioxin-like toxicity/activity in human serum. The method is based on dioxins and DLCs activating the Ah-receptor, which is the main mechanism of action of these compounds. The advantage of measuring activation of AhR compared to measuring concentrations of the different compounds is that the possible supra-additive or antagonistic interactions between the compounds in the mixture can be accounted for(93). The bioassay used in this study was the CALUX® (Chemical-Activated Luciferase gene eXpression) bioassay. It involves dioxins and DLCs activating a firefly luciferase through the AhR pathway gene in cultured H4IIE cells, an enzyme catalyzing the formation of light from ATP and luciferin. The strength of luminescence is linearity related to the amount of activated luciferase, and can be quantified by a luminometer(94). TCDD concentration standards are analyzed at the same time to give a dose-response curve for comparison with the luciferase activity, enabling calculation of TEQ values(Figure 6)(95). However, the activation of the Ah–receptor is not only done by dioxins and PCBs, but also by other substances, such as bilirubin, biliverdin, PAH and flavonoids(63). These can activate the AhR without leading to toxic effects, but still contribute to the TEQ-value if present(96). The samples therefore go through clean-up procedures to separate the contribution from these non-dioxin-like substances and avoid false positives(97).

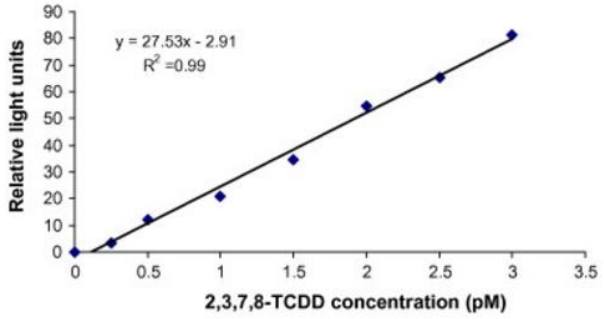


Figure 6: The luminescence from activation of the AhR in H4IIE cells expressed as TCDD-concentrations(93).

The advantage of the CALUX bioassay is that it requires lower volumes of serum compared to gas chromatography/mass spectrometry, it is less time consuming and it is inexpensive(93). Windal et.al.(96) argued that the CALUX bioassay is not a full replacement for GC-MS analysis because CALUX is not determining the toxic equivalency of a complex mixture directly, but only its relative gene induction potency of TCDD. They also observed that often CALUX will give a higher response than what is obtained by chemoanalyses(96). However, several studies have found good recovery and reproducibility and a highly significant correlation between CALUX-derived TEQ and TEQ retrieved from HRGC/HRMS (95, 98).

1.5.3 Evaluating the toxicity of dioxins and DLCs - TEF/TEQ

The large number of congeners of dioxins and dioxin-like substances are not found as single compounds in the environment and human tissues, but rather as mixtures(13). The toxicity of such mixtures is expressed through the Toxic Equivalency (TEQ)(84). The Toxic Equivalency uses the most toxic compound, TCDD, as a reference value and compares other dioxins and DLCs with it(99). Each congener has a Toxic Equivalency Factor (TEF) which indicates the degree of the toxicity compared to TCDD(99). The concentration of the different congeners in a mixture are multiplied with their TEFs and added up, hereby transformed into equivalent concentrations of TCDD(100). To apply the TEF scheme, there must be a common mechanism of action for the compounds involved. For the dioxins and DLCs, binding to the aryl hydrocarbon receptor is the initial step. The TEF is determined from in vivo and in vitro studies and follows many assumptions, the most elementary assumption being that the combination of the congeners are concentration or dose additive(99). The TEF-value

does not give the direct toxic response of a compound, but the ability to bind to the aryl hydrocarbon-receptor, which in turn is associated with toxic effects. TEF can therefore not be used to evaluate effects not mediated through Ah-receptor binding. However, most of the biological effects of these compounds are mediated through the Ah-receptors. To be included in the TEF scheme, a compound must fulfill three criteria: 1) show structural relationship to polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs), 2) it must bind to the ah-receptor and 3) it must mediate biochemical or toxic responses through the Ah-receptor(101). These compounds are the 2, 3, 7, 8-substituted PCDDs and PCDFs and dioxin-like PCBs. Many other substances fulfill the inclusion criteria of the TEQ-scheme, but have insufficient data to define TEF values. The TEF values of the included compounds have been derived through scientific evaluation of all available scientific data(99).

1.6 Epidemiologic study designs

The NOWAC study where our data are extracted from is a population based prospective cohort study. A prospective cohort is a design where a specific group of individuals are randomly selected and followed through time. The individuals are followed up regularly to see if they develop specific outcomes of interest. All the members of the cohort must be at risk of developing the outcome at the start of the study. When experimental studies are excluded because of ethical or practical reasons, observational studies are good alternatives, and the cohort design is considered the best observational design, as seen in the Quality of Evidence pyramid in Figure 7 (102). However, the cases cannot be randomly selected and a major drawback is that the cohort is followed over a longer period of time, and it can lead to a loss-of-follow-up. People in the study can die, migrate or decide to withdraw from the study. This can lead to a selection bias. The design also depends on voluntary participation, which is not randomly distributed in a study group. Health outcomes and exposure under

investigation influences the will to participate. These biases can influence the quality of the collected data (102).

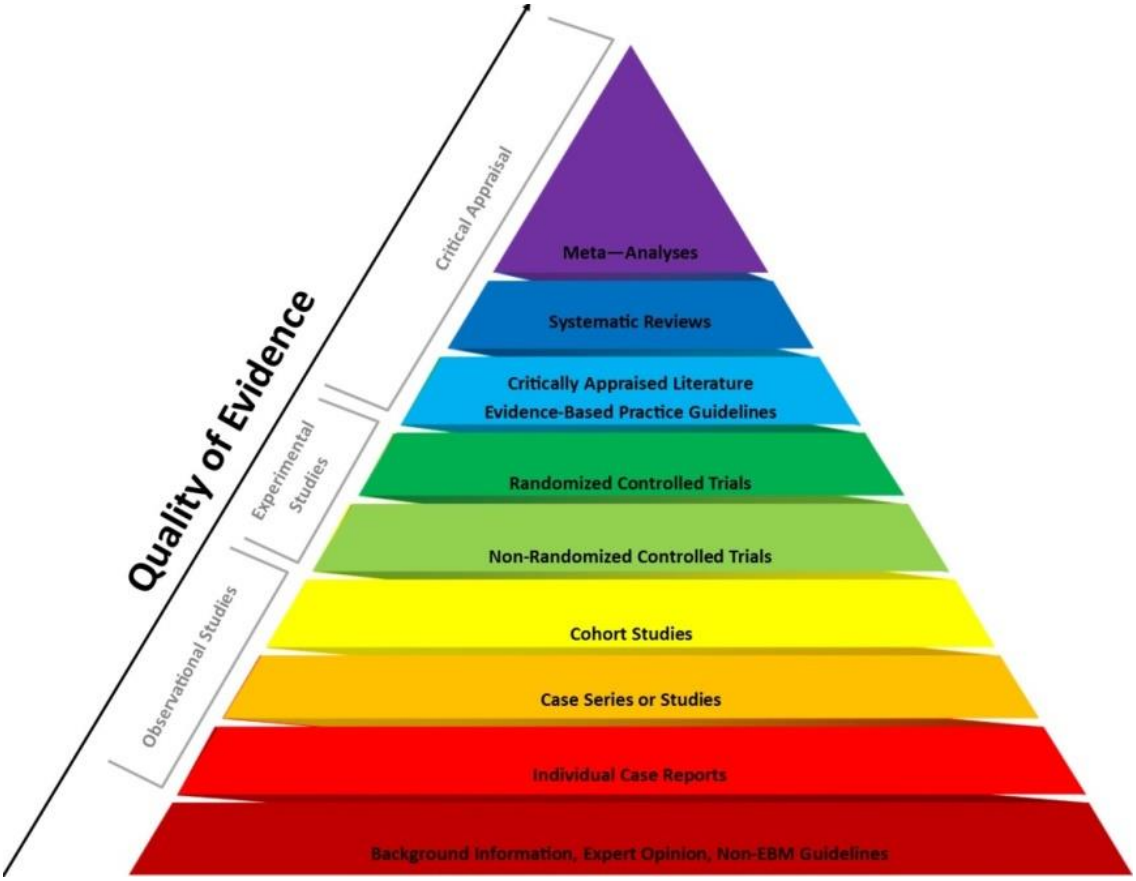


Figure 7: Ranking of study designs based on quality of evidence in the quality of evidence pyramid (94).

In our study, however, it would not be practical to conduct a cohort design. The NOWAC study involves 50 000 women, and it would not be possible to analyze 50 000 blood samples for dioxins and DLCs. The case-control is the second best observational study design as seen in Figure 7. In a case-control study, cases with the outcome under study are compared to controls free of the outcome. A case-control study is by definition retrospective since it starts with the outcome and looks back at exposure. However, in our study women were selected from a cohort to conduct a case-control study, a so-called nested case-control study (103). The advantage of a nested case-control design is that the previous exposure of risk factors can be considered without recall bias, because the information has been obtained in advance by the cohort study. The design is simple, time efficient and has a low cost. The selected cases and controls were compared in regard to exposure of dioxins and DLCs. To avoid confounding bias, the controls were selected to match the cases. In our study, each case was matched to one or two controls in regard to age.

1.7 Statistics

1.7.1 Logistic regression

In our research we wanted to study if there was an association between dioxin-like activity and breast cancer, by evaluating the data acquired from the blood samples in the cases and the healthy controls. If there was a positive association, the women with breast cancer would in average have a higher level of dioxin-like activity in their blood than the controls. Other risk factors also needed to be taken into account, for example smoking, family history of breast cancer and hormone replacement therapy. Since the outcome in this study was binary (getting the disease or not), logistic regression was used.

With logistic regression it is possible to calculate the effect a variable has on an outcome. The dependent variable only has two values, 1= diagnosed with breast cancer, 0 = no breast cancer. In contrast to linear regression, where changes in Y are related directly to changes in X, logistic regression calculates how much the natural logarithm of the odds of Y changes for every unit change in X. It is then possible to calculate the probability that the result = 1 (104). Our cases were matched to the controls in regard to age to control for confounding, and so conditional logistic regression was used. Other important risk factors for breast cancer were also included; age at menarche, parity, age when first child is born, breastfeeding, age at menopause, usage of hormone replacement therapy and oral contraception, BMI, having a mother with breast cancer, alcohol consumption and smoking status. The impact of each variable is calculated and this avoids confounding effects of the other variables(105).

Odds is the probability for an event to happen in relation to the event not happening. The odds ratio is the ratio between two odds(106).

$$Odds = \frac{exposed\ getting\ disease}{exposed\ not\ getting\ disease}$$

$$\text{OR} = \frac{Odds\ exposed\ getting\ disease}{Odds\ unexposed\ getting\ disease}$$

The odds ratio represents the relative risk of developing a disease. An odds ratio greater than 1, means that the risk factor is associated with a higher risk. Is it smaller

than 1, the risk factor is considered a protective factor. If it equals 1, there is no association (102). Thus, if the confidence interval includes 1, that means there is no significant association between variable and outcome.

In our dataset, the variables were both numerical and categorical, and were not normally distributed.

1.8 Aim

The aim of this master thesis was to assess whether dioxin-like activity in blood plasma, measured by the CALUX bioassay, increase the risk of breast cancer.

2. Material and methods

2.1 The study group

The Norwegian Women and Cancer (NOWAC) study was started in 1991 and is a population based prospective cohort study. The main object of NOWAC is to investigate the correlations between internal and external hormones and cancer in women, where breast cancer is the most frequently occurring cancer type. 172 000 women, randomly selected from the Norwegian Central Person Register, in the age of 30-70 years, have answered questionnaires since 1991 and up to 2006(107). The questionnaires handed out included questions involving use of oral contraception and hormone replacement therapy, diet, smoking, alcohol consumption, activity level, reproductive history, anthropometry and family history of cancer. The questionnaires were handed out with an interval of approximately 7 years. The women recruited in 1991-92 could have answered in total three questionnaires, one initial and two follow-ups. In 1998, the study became part of the European Prospective Investigation into Cancer and Nutrition (EPIC), which is a European cooperation between ten European countries(108).

Since 2006, blood samples have been collected from subjects agreeing to it. At the time of blood sampling, detailed questionnaires about current health status were

answered by the women. The Cancer Registry of Norway sends annual updates on which of the study participants have been diagnosed with cancer (107).

All the women participating in NOWAC have signed an informed consent. The participating women are completely anonymous and the researchers cannot connect the results to any individual. The regional ethical committee (REK) for Northern Norway has approved all the analyses, including the blood samples in both the NOWAC study and this particular study(109).

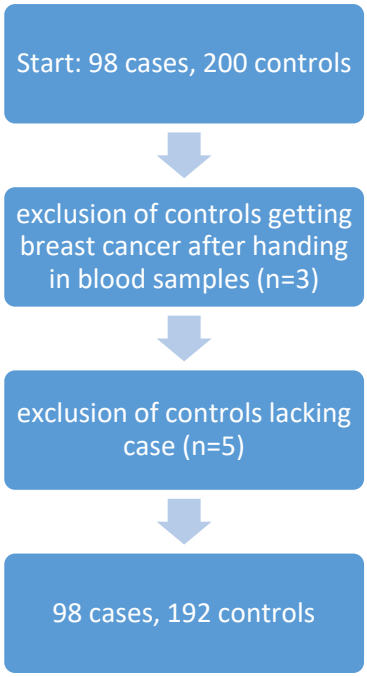
2.1.1 Our study sample

From the NOWAC study, blood samples were selected from 98 women, 71 that were later diagnosed with breast cancer and 27 that had breast cancer already. To each case, two healthy controls were randomly selected, matching the cases on age and year of donating blood samples. In the start of the study, the study sample consisted of 298 women; 98 cases and 200 controls which had never previously been diagnosed with breast cancer. The breast cancer diagnosis of the cases was the first primary invasive cancer they had gotten, primary meaning that the tumour originated from the breast and invasive meant that it infiltrated surrounding tissue or blood. It was possible however that they earlier could have had in-situ breast cancer, borderline tumour in the ovaries or non-melanoma skin cancer. Blood was sampled from the women in 2000, 2001 and 2002. 27 of the cases were diagnosed with breast cancer 1-3 years before or the same year(1999-2002) as handing in their blood samples. The remaining 68 cases were diagnosed 1-6 years after handing in blood samples, between 2000 and 2006(Table 1).

Table 1: Time of diagnosis and sampling of blood of the cases.

	Cases	**Year of diagnosis**	**Time blood sample**
Diagnosed before blood sample	27	1999-2002	0-3 years after diagnosis
Diagnosed after blood sample	71	2000-2006	1-6 years before diagnosis

Of the controls, three women developed breast cancer 7 to 13 years after giving the blood samples. These were excluded from the study. Five more controls were excluded because they were missing matching cases. Summed up, the study sample consisted of 290 women, each case matching with two controls in regard to age and year of blood sampling, a few cases only having one matching control.



2.2 Analysis of blood serum

The samples that were collected from the cases and controls were stored frozen and thawed before preparation. While in the freezer, some of the glass tubes had broken and a few samples were lost in the thawing because of this.

Dioxins and DLCs were extracted from the 298 plasma samples using a liquid-liquid extraction. In short, denatured alcohol was added to the plasma sample and the mixture was extracted thrice with hexane. The organic phases were combined and evaporated under vacuum. The resulting extract was purified on an acid-silica column and reconstituted with 5 µL dimethyl sulfoxide. We determined total DLC

concentrations in samples extracts using an arylhydrocarbon receptor (AhR) reporter gene assay according to the procedure described in (93). The bioassay is based on the expression of the firefly luciferase in H4IIE.Luc cells resulting from the activation of the AhR pathway by dioxins and DLCs. H4IIE.Luc cells (kindly donated by A. Brouwer, BioDetection Systems B.V., Amsterdam, The Netherlands) were obtained by transfecting rat hepatoma H4IIE cells with the luciferase reporter gene plasmid pGudLuc1.1. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents (TCDD-EQ) concentrations in plasma extracts are interpolated from a TCDD standard curve. The limit of detection is 30 pg TCDD-EQ/L, corresponding to approximately 5 pg TCDD-EQ/g lipids

2.3 Exposure variables and covariates

To assess the association between dioxin-like activity in blood and breast cancer, the statistical software Stata 14 was used. Before building the statistical model, the variables needed to be organized. The TEQ-value was considered first by dividing it according to the median. When looking at the histogram of the distribution of the TEQ-values in Figure 8, it is seen that the line at the median (115.0 pg/L) divides the groups so that the TEQ-values in each group would be very similar. Many of the women with low TEQ-values would be considered alongside with women with higher values. Therefore, a group was made where the TEQ-values were divided into quartiles where the 1st to 3rd quartiles where one group, and the 4th quartile accounted for the second group. This is seen as the second line at 209.3 pg/L in Figure 8. This gave a better image of the difference between high and low values of TEQ.

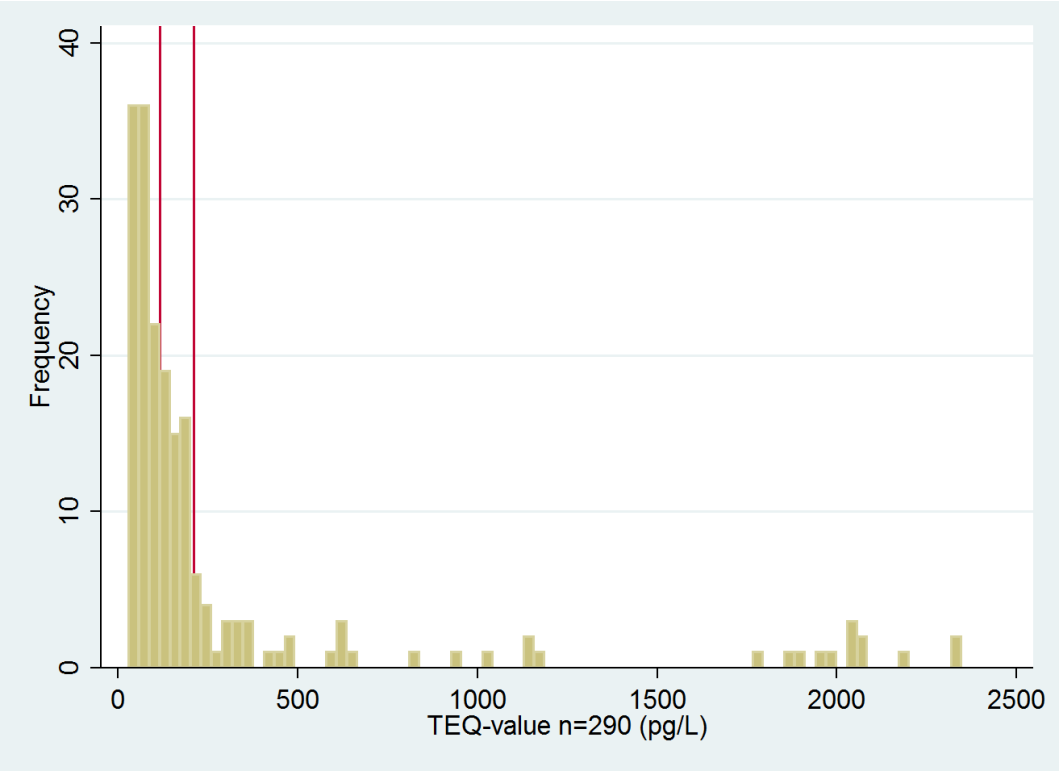


Figure 8: Histogram showing TEQ-values for the cases and controls. The red stripes show the frequencies at the median TEQ-value 115.0 pg/L and 75-percentile TEQ-value at 209.3 pg/L.

Furthermore, 36 women had not answered the question if they had had a mother with breast cancer. These were categorized as not having a mother with breast cancer in the modelling. The menarche category was grouped into above or below 13.2 years, which is the average age of menarche in Norway(110). In this variable two women were missing. The variable "Hormone replacement therapy" was made through using the questionnaire which was handed the women when taking their blood samples, in combination with former questionnaires to obtain the most accurate answers. The menopause group was categorized into three groups, pre, post and others, where others included unknown, hysterectomy and hormone replacement therapy and under 53 years of age. Since all case-control pairs were matched on age, age was not an influencing factor to the model. Alcohol intake was divided according to below or above median (2.0 g/day) of the study group. BMI was divided into three groups; below 25 kg/m^2, 25 to 29.9 kg/m^2 and above 30 kg/m^2, corresponding to normal weight, overweight and obesity respectively (111). Two subjects had missing values in the BMI-group. The age at which the women got their first child was divided according to the median at 24 years. 19 women were missing in this group. Breast feeding was

divided into 0, 1-12 months, and above 12 months. 20 women were missing from this category, but when comparing with the total amount of children-groups, it was seen that the 18 without children belonged to the 20 missing. Therefore, only two women were missing in this group.

2.3.1 Statistical analysis

Univariable conditional logistic regression in Stata 14 was used to obtain the OR for each covariate. This was done using the crude sample where n=290. The result is seen in Table 2. The covariates with p-values significant at 25 %-level were included in a multivariate conditional logistic regression model. These covariates were menopause, parity, mother with breast cancer, BMI grouped in three categories, smoking status and alcohol intake. To work out the most optimal multivariate model, the recipe of logistic regression modelling in Veierøds Medical Statistics, pages 110-114 (112). The six significant covariates were inserted into the model and dropped one by one to find if they were confounding the results. The insignificant covariates were then added back into the model one by one to the model to see if they had an impact.

3. Results

3.1 Study population characteristics

Our study group consisted of 290 women (mean age 48.6 ±3.89 years), 89 of these had been diagnosed with breast cancer. 27 of which were diagnosed before giving blood samples. For each case two age-matched controls were selected. Blood samples were drawn from the participating women, and TEQ-values were obtained. The histograms in Figure 9 show a comparable range of TEQ-values in both the cases and controls.

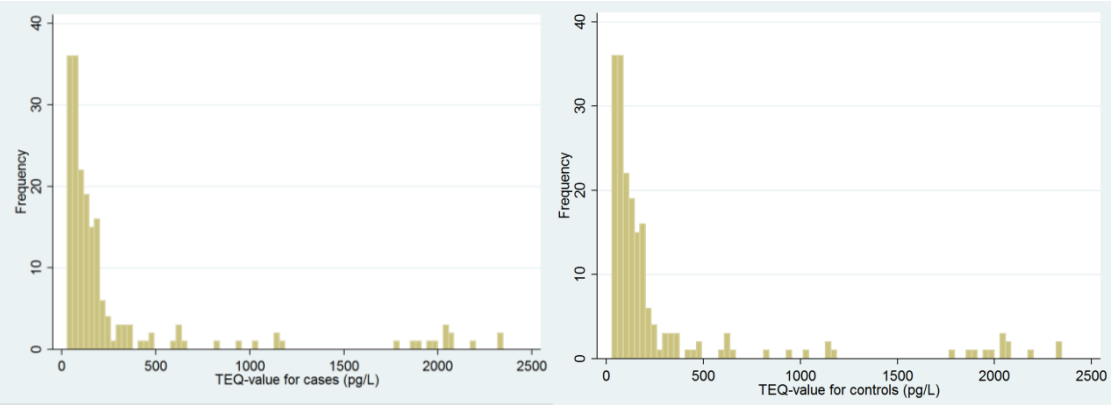


Figure 9: Histogram showing the TEQ-values for cases and controls.

The distribution of cases and controls by age, TEQ-value, age at menarche, parity, age when first child is born, breastfeeding, age at menopause, usage of hormone replacement therapy and oral contraception, BMI, mother with breast cancer, alcohol consumption and smoking status are presented in Table 2. Both the cases and controls had an average menarche age of 13.3 years, had their first child at an average age of 24 years and breastfed for an average of 14.6 months. 33.8% of the women used or had used oral contraceptives and 42.8 % used or had used hormonal replacement therapy. 47.9 % of the women were pre- or perimenstrual and 29.3% were postmenopausal. The mean BMI was 24.7 kg/m^2 in the whole group, and mean alcohol consumption was 3.3 g/day. The TEQ-values did not differ significantly between the cases and controls, as seen both in the histograms in Figure 9 and the numbers in Table 2; the average value was 279.3 pg/L in the cases and 298.7 pg/L in the controls. Furthermore, there was no difference in proportion of people in the 4th quartile of TEQ-values between cases and controls. From Table 2 it is also seen that the highest concentrations of TEQ are among the women in the control group.

Having a mother with breast cancer gave a significantly higher risk of breast cancer; the odds ratio was 3.2 (1,18- 8,87). Furthermore, being a current smoker was associated with a higher breast cancer risk compared to being a non-smoker, with an OR of 1.9 (1,04 - 3,63).

Table 2: Study population characteristics.

Variable	Total(n=290)	Case(n=98)	Control(n=192)	OR(95%CI)	p-value*
TEQ-value (pg/L)					
Mean (std.dev)	292,1 (487,7)	279,3 (433,5)	298,7 (514,2)		
Median(max-min)	115,0(28,1-2346)	111,5(30-2059)	122,7(28,2-2346)		
TEQ-value (pg/g serum lipids)[a]					
Mean	50,4 (84,1)	48,2 (74,7)	51,5(88,7)		
Median(max-min)	19,8 (4,8-404,5)	19,2(5,2-355)	21,2(4,9-404,5)		
75-percentil[b], n (%)					
<209.3 pg/L	218 (75)	71 (72.5)	147 (77.6)	Reference	
≥209.3 pg/L	72 (25)	27 (27.6)	Reference	1,28 (0,73 - 2,23)	0,38
Age at menarche (years)[c], n (%)					
<13,2	161 (55.5)	50(51.0)	111 (57.8)	Reference	
≥ 13,2	127 (43,8)	46 (46.9)	81 (42.2)	1,30 (0,80 - 2,13)	0,30
Age at birth of first child (years)[c], n (%)					
<24	126 (43.5)	44 (44.9)	82 (42.7)	Reference	
≥ 24	145 (50.0)	48 (49.0)	97 (50.5)	0,90(0,54-1,50)	0,70
Parity, n (%)					
0	18 (6.2)	6 (6.1)	12 (6.3)	1,09(0,40-2,97)	0,87
1-2	152(52.4)	46(46.9)	106 (55.2)	Reference	
>2	120(41.4)	46(46.9)	74 (38.5)	1,39(0,85-2,27)	0,19
Breastfeeding (months), n (%)[c]					
0	29 (10.0)	9 (9.2)	20(10.4%)	Reference	
1-12	133(45.9)	43(43.9)	90(46.9%)	1,06(0,45-2,49)	0,89
>12	126(43.5)	46(46.9)	80(41.7%)	1,26(0,53-2,98)	0,60
Menopause, n(%)					
Pre/peri	139(47.9)	50(51.0)	89 (46.4)	Reference	
Postmenopausal	85 (29.3)	24(24.5)	61 (31.8)	0,47(0,20-1,12)	0,09
Others[d]	66 (22.8)	24(24.5)	42 (21.9)	0,92(0,45-1,92)	0,83
Hormone replacement therapy, n(%)					
Never	166(57.2)	58(59.2)	108 (56.3)	Reference	
Former/Current	124(42.8)	40(40.8)	84 (43.8)	0,89(0,52-1,54)	0,68
Oral contraception, n(%)					
Never	98 (33.8)	31(31.6)	67 (34.9)	Reference	
Former/Current	192(66.2)	67(68.4)	125 (65.1)	1,15(0,68-1,92)	0,60
Mother with breast cancer, n (%)					
No	271(93.5)	87(88.8)	184(95.8)	Reference	
Yes	19 (6.6)	11(12.4)	8 (4.2)	3,23(1,18-8,87)	0,02
Body mass index (kg/m^2)[c], n (%)					
BMI<25	181(62.4)	56(57.1)	125 (65.1)	Reference	
BMI 25-29,9	75 (25.9)	31(31.6)	44 (22.9)	1,59(0,89-2,82)	0,09
BMI ≥ 30	32(11.0)	10(10.2)	22 (11.5)	1,19(0,51-2,75)	0,68
Smoking status,n(%)					
Never	99 (34.1)	26(26.5)	73 (38.0)	Reference	
Former	115(39.7)	40(40.8)	75 (39.1)	1,51(0,84-2,71)	0,17
Current	76 (26.2)	32(32.7)	44 (22.9)	1,94(1,04-3,63)	0,04
Consumed alcohol (gr/day), n (%)					
<2.0 gr/day	147(50.7)	56(57.1)	91 (47.4)	Reference	
≥ 2.0 gr/day	143(49.3)	42(42.9)	101 (52.6)	0,69(0,42-1,12)	0,13

*obtained from univariate conditional logistic regression, n=290.

[a]Lipid normalized values obtained by using 0.58% fat content in the blood (113).

[b]values obtained by dividing the TEQ-value according to the 75-percentile

[c]category with missing values

[d]The groups others contain the women that are unknown, got hysterectomy, or uses hormone replacement therapy and are under 53 years of age.

The odds ratios of the TEQ-value obtained from the conditional univariable and multivariable logistic regressions are shown in Table 3. The crude, adjusted and fully adjusted ORs did not differ much and were also not significant in relation to breast cancer. In the multivariable model, having a mother with breast cancer was the only variable significantly associated with breast cancer.

Table 3: Odds ratio and confidential intervals for the TEQ-value with and without adjusting for covariates, obtained from multivariate conditional logistic regression.

TEQ75-value(pg/L)	OR(95%CI)	p-value
Crude	1,28(0,73–2,23)	0,39
Adjusted	1,33 (0,75-2,35)	0,33
Fully adjusted	1,42(0,75 –2,67)	0,28

[a] Not adjusted. n=290.

[b]Adjusted for having a mother with breast cancer. n=288

[b] Complete case analysis n=286. OR adjusted for covariates age at menarche, mother with breast cancer, BMI, smoking status, alcohol consumption, hormone replacement treatment, oral contraception use, age at menopause, breast feeding, age when getting the first child and parity.

Univariate and multivariate conditional logistic regression was done to the study sample after excluding the 27 cases that were diagnosed before blood sampling, and their respective controls (Appendix 1). With the 27 removed, alcohol consumption became a significant risk factor for breast cancer, but the p-value of the TEQ-value did not change significantly.

The TEQ-value was normalized from the wet weight concentration to a lipid weight concentration, to enable comparison with TEQ-values derived in other studies(Table 4) (113). The mean/median lipid weight concentration was 19,8 pg/g serum lipids. Total lipids were not measured in the samples used in this study, instead we used the average lipid concentration (0.58%) in a comparable group of women from NOWAC(113) for the normalization.

4. Discussion

In this study, we assessed the dioxin-like activity in blood from 98 breast cancer cases and 192 age-matched controls. Our study does not support an association between dioxin-like activity and breast cancer. Of the 98 women diagnosed with breast cancer in this study, 27 donated their blood samples 1-3 years after being diagnosed with breast cancer. The other women donated blood up to 6 years prior to breast cancer diagnosis. As one criteria for causality is that the exposure have occurred prior to the outcome, we conducted a sensitivity analysis where these 27 samples and their sub-sequent controls were excluded. This did however not make a difference and gave no significant association between TEQ-values and breast cancer risk. Further, since the cases and controls were matched not only on age, but also on year of blood sampling, we made sure that the declining concentrations of these compounds in human blood over the recent decades did not influence the findings.

Our findings of no association between breast cancer and dioxin-like activity is in agreement with what the majority of other studies have found, such as the meta-analysis conducted by Xu et al. (78) and the case-control study on Norwegian women by Ward et al(81). However, from the literature search that was done in this study, no other studies were found that had investigated the association between dioxin-like activity in blood serum and breast cancer using the CALUX bioassay. Also, many of the other studies have focused on non-dioxin-like PCBs, or only including the PCBs with lower TEF-values. Some also estimated the exposure from questionnaires and geography, being less accurate than measuring the dioxins and DLCs within the individuals.

Having a mother with breast cancer and being a current smoker was found to give an increased risk of breast cancer. These findings are in agreement with other literature(7, 114). However, we did not detect any association between the use of hormone replacement therapy for menopause or parity and breast cancer, risk factors that are well established(7). The reason for this could be that the study sample is relatively small, which may have given a low statistical power. As most study participants have low and comparable TEQ-values, a large study group is required to detect small differences in concentrations between cases and controls.

In a review, TEQ-values from studies that had collected blood samples approximately the same years as us and also used AhR-responsive reporter gene bioassays were compared(Table 4). These studies contained both highly exposed groups and groups from the general population, and some had mixed genders and some only women or only men. When comparing lipid-adjusted TEQ-values, our group has a lower median TEQ-concentration than most of the other studies. This influences the interpretation of the results. If extremely low concentrations were seen, one could expect a different association between TEQ and breast cancer in a highly exposed population, given a linear dose-response relationship between TEQ and breast cancer. Smaller variations make it more challenging to detect an association. However, if the NOWAC women had had very high concentrations compared to other groups, it would be less likely that the dioxin-like activity would have had an effect on breast cancer in a less exposed population. However, if the endocrine disrupting mechanisms of the dioxins and DLCs were actually explained by a non-linear dose-response relationship, e.g exert the most toxic effects at very low and very high concentrations, giving a U-shaped dose-response relationship, the relationship at different concentrations would be more challenging to compare. Also, the effect from the dioxins and DLCs could be present only at very high concentrations. Therefore, our lack of association with breast cancer is not necessarily comparable to other groups with higher concentrations.

Table 4: The lipid-adjusted TEQ-value from our study compared to TEQ-values from other studies (104).

	AhR mediated activity TEQ pg/g serum or plasma lipids						
Study	**Median**	**Range**	**Age(years)**	**Gender**	**Known exposure**	**n**	**Population (year of blood samples collected)**
Our study	19,8	4,8-404,5	41-55	Female	Gp[a]	290	Norwegian women (1999-2006)
Medehouenou et al (93)	9.7	<5–144	18–74	Both	Gp[a]	874	Inuit of Nunavik, Canada (2004)
Long et al (115)	197	38–1188	23–47	Male	Gp[a]	70	Inuit of Greenland (2002-2004)
Dhooge et al (116)	11.9	NA	20–40	Male	Gp[a]	101	Antwerp and Peer, Belgium (1999-2000)
Ayotte et al (117)	93	37–287	25–75	Both	Mixed	40	Coastal communities, Canada (after 1992)
Warner et al (92)	25.4[b]	0–128	20–49	Female	High	32	Seveso, Italy (1998-1999)
Koppen et al (95)	35.0[b]	12–65	50–65	Female	Gp[a]	47[c]	Antwerp and Peer, Belgium (1999)
Pauwels et al (118)	37.4	NA	24–42	Female	Gp[a]	106	Antwerp, Ghent, and Leuven, Belgium (1996-1998)

[a]general population

[b]Arithmetic mean

[c]Pooled samples originating from 200 individual women

4.1 Strengths

The strength in our study is that it is a nested case-control study, the participating women have been followed up through many years so there should be no recall bias as most of the information from the questionnaires was collected prior to breast cancer diagnosis. Also, the majority of blood samples were collected prior to clinical diagnosis, thus we have the possibility to measure the exposure occurring prior to the outcome. The cases are matched to the controls in regard to age and we have also taken into account known risk factors for breast cancer, to avoid confounding of our results

The dioxins and DLCs were measured through the AhR-dependent recombinant bioassay, which measures the dioxin-like activity through activation of the Ah-receptor. Even though this excludes potential effects exerted by the dioxins and DLCs through other pathways, the AhR-pathway is the main mechanism and should be accountable for the dioxin-like toxicity(62). The results are then given as toxic

equivalency factor, in other words, explained as if all the activation was related to the toxicity of TCDD. The advantage with this method is that it does not focus on the concentrations of different congeners, but rather the total effect of all the congeners combined. Some congeners may function as agonists and some as antagonists, and when assessing the activation done by the mixture, it is possible to see the additive effect. This makes more sense than measuring concentrations of compounds, if hypothetically the compounds in the mixture suspected to induce changes leading to breast cancer actually equalized each other, or oppositely, enhanced each other's effects. However, the results can potentially be affected by contamination of natural or synthetic AhR ligands such as bilirubin and flavonoids, and other polyaromatic hydrocarbons. These ligands typically bind to AhR without leading to toxic effects. Most likely, most of them have been removed in the purification of the blood samples in the lab. Several studies have found good recovery and reproducibility and a highly significant correlation between CALUX-derived TEQ and TEQ retrieved from HRGC/HRMS, especially when comparing two groups, such as in our study (95, 98).

4.2 Limitations

Despite not showing any correlations, our study cannot conclude that dioxins and DLCs are not risk factors of breast cancer. The greatest limitation may be the fact that the exposure is measured only a few years before time of diagnosis. We measured the serum levels of dioxins and DLCs in the years 1999-2002, a few years before (and after) diagnosis. Considering the decline in levels of dioxins and DLCs the last decades, it is reasonable to assume that the levels of dioxins and DLCs in the women have been higher when they were younger and exposed to higher concentrations in the environment. It is hard to assess the etiological relevant time for exposure in relation to development of cancer. Cancer is a disease coming from damage to the DNA in a cell, leading to disruption of a cells normal cycle, where the cell can continue to grow and avoid being destroyed. The development of such a disease is complex and factors contributing to the development can be present at different stages and at many years before the cancer can be detected. Measuring the dioxins and DLCs at 1-6 years before (or 0-3 years after) breast cancer was detected, showed that the dioxins and DLCs were present in the women, but not reflecting exposure many years back in time,

possibly at more relevant times. Hence, it is not possible to say anything about exposure at more vulnerable times, such as fetal stage, childhood, puberty or menopause. This is important when considering our lack of association.

In our study, we have focused only on the effects the dioxins and DLCs exert through the Ah receptor, excluding effects from other POPs that could potentially create a harmful cocktail when combined with the dioxins and DLCs. Dioxins and DLCs only constitute a fraction of the total amount of POPs humans are exposed to and it may be that the dioxins and DLCs are more harmful in combination with other POPs. However, it is difficult to assess the effects of many different chemical compounds combined, and our method is good for evaluating the effect the AhR is responsible for.

5. Conclusion

We found no evidence of an association between dioxin-like activity in blood and breast cancer in this study. There was no difference in dioxin-like activity in blood between cases and controls. This finding is in line with many studies, although no studies equal to ours. Leaving out the 27 cases that were diagnosed before giving blood samples did not make a difference on the results. We did however find a significant association between smoking and breast cancer, and having a mother with breast cancer and breast cancer. No associations were found between other known risk factors and breast cancer. It was also seen that the participants in this study had lower lipid-adjusted TEQ-concentrations than those in many other studies. Our study is a snapshot of the dioxin-like activity and breast cancer. For these reasons, this study does not rule out the possibility of there being an increased risk of breast cancer.

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Appendix 1

Table 5: ORs from univariable conditional logistic regression, cases diagnosed before blood sampling excluded, n=263

	OR(95% CI)	p-value[a]
TEQ75, quartiles[b]		
1st-3rd	Reference	1,0
4th	1,25 (0,65 – 2,39)	0,67
Age at menarche, years[c]		
<13,2	Reference	1,0
≥13,2	1,05 (0,58 – 1,88)	0,88
Age at birth of first child[c], years		
<24	Reference	1,0
≥24	0,85 (0,47 – 1,53)	0,59
Parity, number of children		
0	0,80 (0,21 – 3,01)	0,74
1-2	Reference	1,0
>2	1,57 (0,89-2,76)	0,12
Breast feeding[c], months		
0	Reference	1,0
1-12	1,45 (0,50 – 4,21)	0,49
<12	1,89 (0,64 – 5,58)	0,25
Menopause		
pre/peri	Reference	1,0
Post	0,97 (0,34 – 2,53)	0,88
Others[d]	0,80 (0,34 – 1,85)	0,60
Hormone replacement therapy		
Never	Reference	1,0
Former/current	0,79 (0,42 – 1,48)	0,46
Oral contraception use		
Never	Reference	1,0
Former/current	1,25 (0,68 – 2.32)	0,47
Mother with breast cancer		
No	Reference	1,0
Yes	3,84 (1,16 – 12,70)	0,03
Body mass index[c], kg/m2		
<25	Reference	1,0
25-29,9	1,78 (0,92 – 3,46)	0,09
≥30	1,23 (0,43 – 3,53)	0,70
Smoking status		
Never	Reference	1,0
Former	1,56 (0,78 – 3,13)	0,21
Current	2,10((0,99 – 4,45)	0,05
Alcohol intake, gr/day		
<2	Reference	1,0
≥2	0,48 (0,27 – 0,87)	0,02

[a]obtained from univariate conditional logistic regression, n=263.

[b]values obtained by dividing the TEQ-value according to the 75-percentile

[c]category with missing values

[d]The groups others contain the women that are unknown, got hysterectomy, or uses hormone replacement therapy and are under 53 years of age.

Table 6: Odds ratios and confidential intervals obtained from multivariate conditional logistic regression, cases diagnosed before blood sampling excluded, n=263. Only alcohol groups were significant variables.

TEQ75-value(pg/L)	Crude OR(95%CI)	Crude p-value
Crude[a]	1,25 (0,65-2,39)	0,50
Adjusted[b]	1,18 (0,61–2,27)	0,63
Fully adjusted[c]	1,25 (0,58-2,70)	0,57

[a] Not adjusted for any covariables.

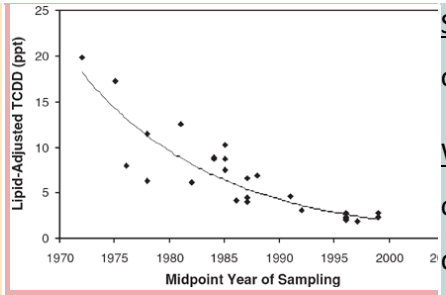
[b]Adjusted for the only variable being significant in the multivariate regression; alcohol groups.

[c] Complete case analysis n=286. OR adjusted for covariates age at menarche, mother with breast cancer, BMI, smoking status, alcohol consumption, hormone replacement treatment, oral contraception use, age at menopause, breastfeeding, age when getting the first child and parity. n=244.

Summary of article evaluations

Reference: Aylward, L. L. and S. M. Hays (2002). "Temporal trends in human TCDD body burden: Decreases over three decades and implications for exposure levels." journal of Exposure Analysis and Environmental Epidemiology **12**: 319-328.

GRADE	
Documentation	IV
Recommendation	C

Aim	**Material and methods**	**Results**	**Discussion/comments**
Estimate historical (ca. 1970), current, and likely future of "background" TCDD body burdens in the general population. **Conclusion** Mean lipid levels of TCDD exhibited a steady decrease by nearly a factor of 10 over 30 years from 1970 to 2000. **Land** The United States, Canada, Germany, and France **Year of sampling data** 1971-1999	Study design: Unsystematic literature review Method: They surveyed literature for studies reporting levels of TCDD in fat tissue or blood samples of the general populations. The data from the various studies were plotted versus the median year of sampling to evaluate the temporal trend in lipid-adjusted TCDD levels in the general population. Modelling was performed for changes in body burden as a function of intake.	 Mean lipid-adjusted TCDD levels from general population samples in the US, Canada and western Europe.	Strengths: Consistent findings from study groups involving over 2800 individuals. Weaknesses: There is no information on how they have conducted the literature search. The population of the different studies have not been defined. A lack of data on total TEQ body burdens from before the 1980s. The data compiled and analyzed do not constitute a statistically representative sampling of general population body burdens over time. The data are samples of opportunity collected in various locations and for various purposes, and they represent groups of different sizes, predominantly from males.

Reference: Warner, M., et al. (2005). "Dioxin-Like TEQ of women from the Seveso, Italy area by ID-HRGC/HRMS and CALUX." journal of Exposure Analysis and Environmental Epidemiology **15**: 310-318.

GRADE	
Documentation	III
Recommendation	B

Aim

Characterize current background exposure to dioxins and DLCs in the Seveso area of Italy, and examine the correlation of these measurements with the CALUXs by XDS bioassay measure of TEQ with high resolution gas chromatography/mass spectrometry.

Conclusion

The two measures were not significantly correlated. More validation of the CALUX bioassay with larger sample volume is needed before application as an exposure measure in large-scale epidemiologic studies of health effects of dioxin-like compounds.

Material and methods

Study design: Cross sectional

Reruitment of participants: Participants were women from 20 to 49 who participated in an endometriosis case-control study at Desio Hospital, Italy, about 25km north of Milan. These women have been and are exposed to significant amounts of TCDD. A total of 78 women participated.

Criteria of inclusion: the participating women had to be 20–50 years old and scheduled to undergo laparoscopy for pelvic pain, infertility, tubal ligation, or adnexal/uterine mass at the Hospital of Desio, Italy between July 1998 and December 1999.

Method: The women were interviewed by trained nurse-interviewers about sociodemographics, personal habits, and reproductive history. A 70-ml blood sample

Results

Main findings: Dioxin and DLC concentrations in the women from the Seveso area were comparable to body burdens reported in other background-exposed populations in industrial nations. Also, the distribution of congeners was similar to that reported for other North American and European countries. No significant correlation between CALUX-TEQ and total TEQ or any TEQ measure derived from HRGC/HRMS data was found.

Other findings: Age was a significant predictor of Total TEQ in this Italian sample, consistent with that reported by others.

	Full sample (n=32)			Excluding non-detects (n=22)		
	TEQ Mean (range)	CALUX total TEQ *versus* HRGC/HRMS R_s (P-value)	CALUX DOX fraction TEQ *versus* HRGC/HRMS R_s (P-value)	TEQ Mean (range)	CALUX total TEQ *versus* HRGC/HRMS R_s (P-value)	CALUX DOX fraction TEQ *versus* HRGC/HRMS R_s (P-value)
CALUX TEQ[a, b]						
Total TEQ	25.4 (0–127.6)			37.0 (1.6–127.6)		
DOX fraction TEQ	21.2 (0–67.3)			30.8 (1.6–67.3)		
HRGC/HRMS-Derived-TEQ						
PCDD TEQ	7.6 (0.1–32.0)	0.34 (0.06)	0.34 (0.06)	8.2 (0.1–32.0)	0.33 (0.13)	0.34 (0.12)
PCDF TEQ	7.1 (3.6–17.1)	0.25 (0.17)	0.25 (0.17)	7.4 (3.6–17.1)	0.15 (0.50)	0.16 (0.48)
cPCB TEQ	8.1 (2.6–36.7)	-0.18 (0.34)	-0.16 (0.37)	7.3 (2.6–14.9)	-0.17 (0.45)	-0.15 (0.52)
ortho PCB TEQ	8.3 (0–73.7)	-0.21 (0.25)	-0.20 (0.28)	9.0 (0–73.7)	-0.25 (0.25)	-0.23 (0.30)
PCDD/PCDF TEQ	14.7 (5.2–49.1)	0.31 (0.09)	0.31 (0.08)	15.6 (5.2–49.1)	0.24 (0.28)	0.24 (0.28)
PCDD/PCDF/cPCB TEQ	22.8 (9.1–64.0)	0.07 (0.70)	0.08 (0.67)	22.9 (9.1–64.0)	0.04 (0.88)	0.05 (0.82)
Total TEQ	31.2 (12.7–88.3)	0.04 (0.82)	0.05 (0.78)	31.9 (12.7–88.3)	0.001 (0.99)	0.02 (0.93)

[a]CALUX DOX fraction: % detected=22/32=69%, average DL=15.2 ppt, lipid-adjusted.

[b]CALUX PCB fraction: % detected=2/32=6%, average DL=26.7 ppt, lipid-adjusted.

Discussion/comments

Check list cross sectional study:

Was the population well defined? yes
Was the study group representative for the population? It was representative for women of the age 20-49 years in the same area.
Is it explained if and how the responders differ from the non-responders? No
Is the answering percentage high enough? No data given.
Was the data collection standardized? Yes
Are objective criterias used for evaluating outcomes/exposure? Yes, data from blood sample analyzes.
Have adequate methods been used in the data analysis? Yes. HRGC/HRMS analyzes and bioassays were performed on an independent basis.

Strengths: The study uses both the golden standard HRGC/HRMS to assess the TCDD levels in the blood serum, as well as a bioassay. Extensive cleanup methods were used before analyzes.

Weaknesses: A relatively low study sample. Not studying women older than 49 years old.

Land Italy **Year of sampling data** 1998-1999	was taken. 15 mL of this was examined by HRCG/HRMS. 32 plasma samples (4ml) were sent to XDS CALUX bioassay analysis. XDS was blind to the HRGC/HRMS results.		What did the writers discuss? That their findings of the CALUX only have a moderate sensitivity (20–54%) and that larger sample volumes may be necessary in the analyses. They argue that others have done a less thorough cleanup of the samples than them, possibly yielding a falsely higher TEQ and sensitivity. Other literature supporting their findings? Several studies supported the findings of TCDD-levels, also compared to age, but not supporting their findings in bad correlations between HSGC/HSMS.

Reference: Nøst, T. H., et al. (2013). "Persistent Organic Pollutants in Norwegian Men from 1979 to 2007: Intraindividual Changes, Age–Period–Cohort Effects, and Model Predictions." Environmental Health Perspectives **121**(11-12): 1292-1298.

GRADE	
Documentation	III
Recommendation	C

Aim

Examine the association between individual serum TCDD levels and breast cancer risk in women exposed to high levels of TCDD after an industrial explosion in 1976 in Seveso, Italy.

Conclusion

Individual serum TCDD is significantly related Individual serum TCDD is significantly related with breast cancer incidence among women in the SWHS cohort.

Land

Italy

Year of sampling data

Material and methods

Study design: Nested case-control

Recruitment of participants: Data from the Seveso Women's Health Study (SWHS), a historical cohort study of the female population residing around Seveso at the time of the explosion in 1976, was used. The 981 women were infants to 40 years old in 1976, had resided in one of the most highly contaminated zones, A or B, and had adequate stored sera collected soon after the explosion.

Method: An interview was conducted by a trained nurse-interviewer who was blinded to serum TCDD levels and zone of residence. Blood serum samples stored since 1976 were measured by high resolution mass spectrometry. For women with serum samples collected after 1977, the serum TCDD level was back extrapolated using a first-order kinetic model, assuming a 9-year half-life.

Statistical analyses were performed using STATA 7.0

Results

More than a 2-fold increase in the hazard rate associated with a 10-fold increase in serum TCDD.

Table 3. Results of Cox proportional hazards model for association between lipid-adjusted serum TCDD levels and female breast cancer risk, SWHS, Italy.

Exposure	Cases/total	Crude hazard ratio (95% CI)	*p*-Value
Log_{10} TCDD[a] (ppt)	15/981	2.1 (1.0–4.6)	0.05
TCDD (ppt)			
< 20	1/156	1.0	
20.1–44	2/241	1.0 (0.1–10.8)	
44.1–100	7/249	4.5 (0.6–36.8)	
> 100	5/335	3.3 (0.4–28.0)	0.07[b]

[a]Hazard ratio for 10-fold increase in serum TCDD concentration. [b]Test for trend.

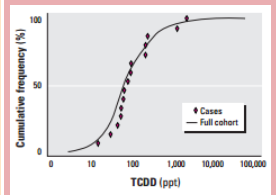


Figure 1. Cumulative distribution of 1976 serum TCDD levels for breast cancer cases ($n = 15$) versus full cohort ($n = 981$), SWHS, Italy.

The median decreases in summed serum POP concentrations (lipid-adjusted) in 1986, 1994, 2001, and 2007 relative to 1979 were –22%, –52%, –54%, and –68%, respectively. Substantial declines in all POP groups with the exception of chlordanes were observed. Time period (reflected by sampling year) was the strongest descriptor of changes in PCB-153 concentrations. Predicted PCB-153 concentrations were

Discussion/comments

Check list:

1. The exposed and non-exposed were comparable in relation to important background factors.

2. The exposed individuals were representative for women being infants to 40 years old in 1976.

3. The non-exposed group was selected from the same population as the exposed group.

4. The study was mostly not prospective, except some back extrapolations.

5. Exposure and outcome was measured in the same way in the two groups.

6. 981 women were followed, 15 with breast cancer, a little too few cases.

7. A dropout analysis is done.

8. The follow-up period could be longer.

9. Confounders have been made account for.

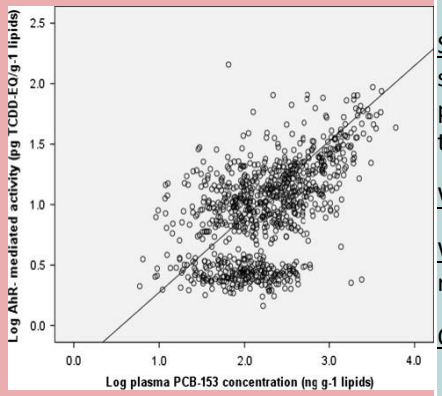
10. The interviewer was blinded to serum TCDD levels and zone of residence. Medical records were obtained and reviewed by a cancer pathologist who was blinded to the woman's exposure.

Strengths: The relationship between serum TCDD concentration and breast cancer incidence was examined, thus eliminating potential bias associated with disease survival. In addition, they collected information in an interview, allowing consideration of potential confounding by known risk factors in the analysis. Finally, they measured individual serum TCDD

1976-1981 (a few in 1996-97)		consistent with measured concentrations in the study population.	concentrations near the time of exposure, thus minimizing exposure misclassification. Weaknesses: small number of breast cancer cases. No other studies on the same study groups show correlations between breast cancer and TCDD.

Reference: Medehouenou, T. C., et al. (2010). "Determinants of AhR-mediated transcriptional activity induced by plasma extracts from Nunavik Inuit adults." Chemosphere **80**(2): 75-82.

GRADE	
Documentation	III
Recommendation	B

Aim	**Material and methods**	**Results**	**Discussion/comments**
Obtain a global measure of persistent organic pollutants(POPs) in Inuit people by assessing AhR-mediated transcriptional activities in blood serum samples. **Conclusion** AhR activity increases with incremental exposure to contaminant from the marine food chain in the Inuits. **Land** Canada **Year of sampling data** 2004	Study design: Cross-sectional study Reruitment of participants: permanent Inuit residents of Nunavik aged 18 years and older. A stratified random sampling of private Inuit households with the community being the stratification variable was used to obtain a standard representation of the target population. In total, 874 Inuits were included. Method: Several self-administered and interviewer-completed questionnaires were used to obtain information regarding demographics, lifestyle habits, nutrition and health indicators. Blood samples and anthropometric measures were taken. The blood serum was assessed using DR-CALUX and GC/MS. SAS Software was used to perform all the statistical analyses.	The geometric mean AhR-mediated activity expressed as TEQ was 8,9 pg/g lipids (range <5-144 pg/g lipids). PCB-153 concentrations measured by high-resolution GC/MS was moderately correlated to AhR-mediated activity (Pearson's R=0,53, p<0,001). Multiple linear regression analyses revealed that age and omega-3 fatty acids in erythrocyte membranes(an index of marine food consumption) were positively associated with plasma-AhR-mediated activity (p<0,001), whereas a negative association was noted with body fat mass (p=0,037) Figure: Correlation between AhR-mediated transcriptional activity in plasma extracts and plasma PCB-153 concentration in Inuit adults from Nunavik.  	Check list cross sectional study: 1. *Was the population well defined?* Yes 2. *Was the study group representative for the population?* Yes 3. *Is it explained if and how the responders differ from the non-responders?* No 4. *Is the answering percentage high enough?* 50 %, a little low. 5. *Was the data collection standardized?* Yes. 6. *Are objective criterias used for evaluating outcomes/exposure?* Yes. 7. *Has adequate methods been used in the data analysis?* Yes, both bioassay and GC/MS is used. Strengths: obtained both AhR-mediated activity and concentrations of specific POPs in blood serum as well as questionnaires. Inclusion of a plasma standard allowed documenting the precision and accuracy of the bioassay. Large study group. Weaknesses: only 50 % answered. What did the writers discuss? The advantage of AhR, the difference in results in other studies Other literature supporting their findings? Yes.

Reference: Recio-Vega R, Velazco-Rodriguez V, Ocampo-Gomez G, Hernandez-Gonzalez S, Ruiz-Flores P, Lopez-Marquez F. Serum levels of polychlorinated biphenyls in Mexican women and breast cancer risk. J Appl Toxicol. 2011;31(3):270-8.	**GRADE**	
	Documentation	III
	Recommendation	C

Aim	**Material and methods**	**Results**	**Discussion/comments**
Evaluate the relation between polychlorinated biphenyls (PCB) exposure and breast cancer risk in Mexican women **Conclusion** They showed an association between heavy and potentially estrogenic PCB congeners and breast cancer risk. **Land** Mexico **Year of sampling data** Not given	Study design: case-control Participants: 140 women aged 25-80 years residing in Comarca Lagunera, Mexico. 70 of which were newly diagnosed with breast cancer, identified by biopsi. The 70 controls were women with biopsies negative for malignancies. Method: blood was sampled and measured for 20 PCB congeners, both non-DL and DLC-PCBs, using GC-electron capture detection. Information regarding sociodemographic variables and status, reproductive history,lifestyle factors, family history of cancer, occupational history and diet. Potential sources of PCB located close to the womenshomes were registered. The PCBs were divided in 5 groups according to structure-related activity.	For the total population, PCBs in groups 2b(congeners 128, 138, 170), 3(153, 180) and 4(8, 195, 206, 209) and total PCBs were significantly associated with breast cancer. For premenopausal women, only group 4 was significant. For postmenopausal women, PCB groups 1a(44,52), 2b and 4 and total PCBs were significantly associated with breast cancer.	Check list case-control study 1. Were the cases and controls selected from and comparable to the same population? Yes. 2. Are the groups comparable in relation to important confounders? Yes 3. Is the disease of the cases sufficiently explained? Yes, identified by biopsies. 4. Is it clear that the controls did not have the disease? Yes. 5. Did the authors consider important confounders in the study design and/or analysis? Yes 6. Is the exposure measured and graded in the same way in cases and controls? Yes. 7. Was the one measuring the exposition blinded for who were cases and controls? No. 8. Was the response rate high enough in both groups? Not stated. Strengths: They have taken into account all important risk factors. Confirmed the diagnosis by biopsies. Measuring PCBs using GC/ECD. Weaknesses: small groups. What did the writers discuss? Appropriate times for measuring the exposure, their findings compared with other studies, the change in exposure of the individuals, the role PCBs play in cancer development, and the toxicity of the OCB congeners related to chlorination, and their findings of relation of known risk factors and breast cancer. Other literature supporting their findings? Our data are consistent with some but not all published studies in which the general population with relatively low levels of environmental exposure has been assessed. They assess these inconsistencis to origin from differences in the groups in the different studies.