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MiniReview

Biomonitoring and Hormone-Disrupting Effect Biomarkers of Persistent Organic Pollutants *In Vitro* and *Ex Vivo*

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Abstract: Persistent organic pollutants (POPs) include lipophilic legacy POPs and the amphiphilic perfluorinated alkyl acids (PFAAs). They have long half-lives and bioaccumulate in the environment, animals and human beings. POPs possess toxic, carcinogenic and endocrine-disrupting potentials. Endocrine-disrupting chemicals (EDCs) are compounds that either mimic or block endogenous hormones and thus disrupt the normal hormone homeostasis. Biomonitoring assesses the internal doses of a person to provide information about chemical exposures. Effect biomarkers assess chemicals potential to affect cellular functions *in vivo*/*ex vivo*. Human beings are exposed to complex mixtures of chemicals, having individually very different biological potentials and effects. Therefore, the assessment of the combined, integrated biological effect of the actual chemical mixture in human blood is important. *In vitro* and *ex vivo* cell systems have been introduced for the assessment of the integrated level of xenobiotic cellular effects in human beings. *Ex vivo* studies have shown geographical differences in bioaccumulated POP serum levels, being reflected by the combined biomarker effects of the complex mixture extracted from human serum. Xenohormone receptor transactivities can be used as an *ex vivo* integrated biomarker of POP exposure and effects. Epidemiological and *in vitro*/*ex vivo* studies have supported the potential impact of the combined effect of serum POPs on the activity of hormone and/or dioxin receptors as a risk factor for human health. With focus on hormone disruption, this MiniReview will give an update on recent POP-related endocrine-disrupting effects *in vitro*/*ex vivo*/*in vivo* and some related genetic data.

Persistent organic pollutants (POPs) are ubiquitous and bioaccumulate in the environment, in wildlife, up through the food web and human beings. The POPs often have long half-lives up to 5–15 years in human beings [1] and for most of these persistent compounds, the bioaccumulation in an organism increases with age. Other chemicals found in the environment are readily metabolized and released from the body, but when the exposure to these non-persistent compounds is continuous, their effect might mimic the effect of POPs. Biomarkers of exposure reflect emission, levels in the environment, and internal and biologically effective doses. Biomarkers of effects are early markers on changes and disruption upon exposure, and genetic biomarkers might elucidate the susceptibility of an individual. Thus, an integrated biomonitoring of exposure, biomarkers of effect and genetic can reflect the susceptibility and health risk of an individual [2,3]. Some POPs are classified as endocrine-disrupting chemicals (EDCs) that are compounds that can mimic, interfere or block the function of endogenous hormones and thereby disrupt the normal hormone homeostasis of the body. The European Commission has defined EDCs as 'an exogenous substance or mixture that alters the function (s) of the endocrine system, and consequently causes adverse health effects in an intact organism or its progeny or (sub)-population' [4]. Growing evidence shows that EDCs may also modulate the activity and/or expression of steroidogenic enzymes, having the ability to convert circulating precursors into active hormones [5].

Many EDCs are known to act as agonists/antagonists of the oestrogen (ER), androgen (AR) and aryl hydrocarbon receptor (AhR), to interfere with thyroid hormone (TH) function and to interfere with the steroid enzymes such as aromatase enzyme that convert testosterone to E2 (17β-estradiol) [5–8]. Moreover, because of the cross interaction of the AhR with the ER and AR [9] and TH [10], the effects of EDCs on AhR can help to elucidate the cellular mechanisms behind hormone disruption [11,12]. The interference of EDCs with endogenous hormones is of particular concern for the developing organism as it is highly sensitive to hormonal changes. These hormonal interferences can result in changes that are permanent and first show up in the adult life [5]. The broad categories of human health effects that may be linked to exposure to environmental contaminants include the following: birth defects, decreased fertility, altered sex hormone balance, immune system defects, neurological effects such as reduced IQ and behavioural abnormalities, altered metabolism, cancer and specific organ dysfunctions [5].

Some EDCs occur naturally (e.g. phytoestrogens), whereas others are industrial chemicals such as bisphenol A and other

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phenols, and plasticizers like phthalates and adipates commonly used in the plastic industry [2,3]. The EDCs also include the lipophilic legacy POPs, including polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins/furans (PCDDs/PCDFs), organochlorine pesticides (OCPs) and polybrominated flame-retardants. The lipophilic legacy POPs biomagnifies through the food chain and are found at high concentrations in fatty tissues of predatory fish and birds, seals, whales and polar bears [13]. Due to long-range transport by atmospheric and oceanic currents [14,15], the human exposure is not limited to individuals living close to the sources of the contaminants, and thus Arctic populations such as the Greenlandic Inuit display very high body burden of POPs [16]. The legacy POPs, with half-lives between 5 and 15 years [1], have been through global and regional conventions with the goal of eliminating or reducing emissions [17]. The temporal decreasing trend seen for many legacy POPs indicates that the present day contamination is largely a 'legacy' of past releases of these chemicals [18,19].

Another POP group is the perfluorinated alkyl acids (PFAAs), a large group of chemicals used since the 1950s in different industrial and commercial applications (e.g. 'non-stick cookware', carpets, furniture and food stuff packing). For a long time, they were considered metabolically inert and non-toxic [20]. However, the carbon-fluorine bond renders these chemicals very resistant to biodegradation, and the transformation or biodegradation of precursor PFAAs is suggested to occur via both abiotic and biotic degradation pathways, where perfluorooctane sulphonate (PFOS) and perfluorooctanoic acid (PFOA) are typical final degradation products [21,22]. The PFAAs are persistent with half-lives between 4 and 10 years [23], they bioaccumulate in the environment, animals and human tissues and are globally distributed [21,22,24]. Unlike the legacy POPs that accumulate in lipid-rich tissues, the PFAAs bind to blood proteins and accumulate mainly in liver, kidney and bile secrets [25].

With focus on hormone disruptive potentials, the present MiniReview aims to give a short overview of legacy POP and PFAA exposures and biomarkers endocrine-disrupting effects. Biomonitoring of Legacy POPs in the Arctic updates recent epidemiological studies including biomarkers of exposure and effects biomarkers of legacy POPs *ex vivo* and genetic sensitivity in some arctic populations. Biomonitoring of Exposure and Effect Biomarkers of PFAAs gives an overview of recent epidemiological studies on PFAA exposures, effect biomarkers and genetic sensitivity biomarkers followed by supporting *in vitro* data including some oxidative stress data.

Biomonitoring of Legacy POPs in the Arctic

The levels and trends of legacy POPs in the Arctic have been assessed since 1997 by the Arctic Monitoring and Assessment Programme (AMAP) and three reports on POP biomonitoring, toxicological effects and their health risks for Arctic populations were published in 1998, 2002 and 2009 [26–28]. Since 2000, parallel studies have been carried out in Greenland on the human monitoring of biomarkers for POP exposure and biomarkers of POP effects, focusing on hormone disruptive potentials and some genetic sensitivity biomarkers [29].

The Arctic populations have some of the highest body burden of POPs [16] that in some Greenlandic districts exceed the Level of Concern guideline suggested by Health Canada [29,30]. The levels of POPs in Arctic Inuit correlate with age, smoking and the level of n-3 polyunsaturated fatty acids in the plasma, the latter being strong indicators of the main source of POP contaminants in their traditional marine food [29,31–34]. In Greenland (fig. 1), regional and gender (highest in men) differences in serum POP levels are observed, with the highest contaminant levels found in Inuit living at the east coast and at the north-west (Qaanaaq, although at a lower level) [29,35,36] (fig. 2) with higher levels of PCBs, *p*,*p*′- DDE and *p*,*p*′-DDT in marine species and birds compared to the west coast [37,38]. Some districts and settlements such as Ittoqqortoormiit (east coast) still primarily rely on traditional foods, whereas the diet in Qeqertarsuaq (west coast) and Narsaq (south) is more westernized [29,35,39]. A time trend analysis of POPs revealed a decrease in atmospheric emission (modelled), ringed seals and human body burden since the late 1990s to 2010 [Bonefeld-Jorgensen EC, Krüger T, Riget F, Skov H, Christensen JH, Hansen KM *et al.,* in preparation]. For both ringed seals and human beings, the decrease trend for legacy POPs was higher at the west coast (Nuuk > Disko Bay) compared to the east coast (Ittoqqortoormiit), whereas the level of methyl mercury and selenium for human beings was highest in north-west (Qaanaaq), and no decreasing trend was observed. However, a significantly increased trend for selenium was seen in the Disko Bay and Ittoqqortoormiit, for human beings and seals respectively [Bonefeld-Jorgensen EC, Krüger T, Riget F, Skov H, Christensen JH, Hansen KM, in preparation]. The decreasing time trends of legacy POPs might be a combination of legislation and change to a more western diet.

As a supplement to human POP biomonitoring studies, a physiologically based pharmacokinetic (PBPK) model was set up to estimate the fate of POPs in liver, blood, muscle and adipose tissue of Greenlandic Inuit, following long-term exposure to traditional Greenlandic diet [40]. The PBPK model described metabolism, excretion and accumulation on the basis of their physicochemical properties and metabolic rates of POPs in the organisms. Basic correlations between chemically analysed blood POP concentrations and calculated daily POP intake from food questionnaire were conducted for Greenlandic Inuit from four cities in West Greenland (collected from 2003 to 2006). Significant correlations were found between the blood POP concentrations and the calculated daily intake of POPs for Inuit from several districts. Despite the large variation in circulating blood POP concentrations, the PBPK model predicted blood concentrations of a factor 2–3 within the actual measured values. Moreover, the PBPK model showed that estimated blood POP concentration increased significantly after consumption of meals. As expected, for individuals who had a high internal burden of POPs, the estimated blood levels were less influenced by recent meal intake. The model data also indicated that, of the POPs accumulated in the body, the concentrations were highest for PCB153. Fur-

Fig. 1. Map of Greenland. The map shows the districts included in the studies presented in the review.

thermore, the model estimated a significant internal body POP burden several years after the mentioned dietetic shift from traditional to westernized food, and that contaminant accumulation was two to six times faster than the decay after a shift to a diet low in contaminants [40]. On the basis of these results, we suggest that PBPK modelling is implemented as a tool in future human health exposure and effect assessments in the Arctic.

Biomarkers of hormone-disruptive effects of legacy POPs ex vivo.

Today, it is well known that the level and profiles for the various POP groups vary among Greenlandic districts (fig. 2) [29]. Studies on biomarkers of toxicological effects have shown that the individual POPs have very different biological potentials. For example, some PCB congeners possess an oestrogenic potential (e.g. some hydroxy-PCBs), whereas others are anti-oestrogenic (e.g. PCB153, PCB180, PCB138) and anti-androgenic (PCB138); in addition, some have dioxin-like potentials (e.g. PCB126). Likewise, for OCPs, there has been reported both oestrogenic potentials [e.g. toxaphene, β-hexachlorocyclohexane (β-HCH), dichlorodiphenyltrichloroethane (DDT) and 1,1-Dichloro-2,2-bis(p-chlorophenyl) ethylene

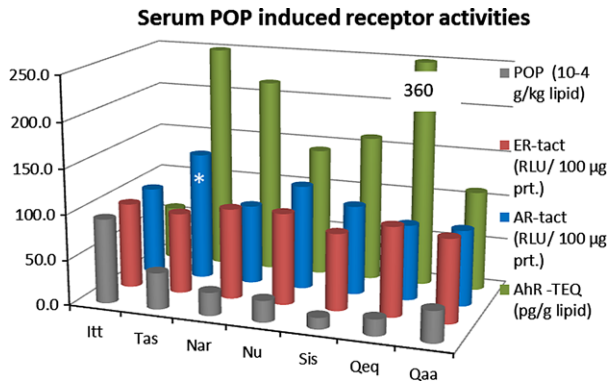

Fig. 2. Serum POP induced receptor activities in Greenlandic Inuit [36,45–47,50,51]. POP: persistent organic pollutant, including polychlorinated biphenyls and organochlorine pesticides. ER-tact: oestrogen receptor transactivity induced by serum extract of lipophilic legacy POPs in the presence of the ER agonist 17β-estradiol (E2). AR-tact: androgenic transactivation induced by serum extracts of lipophilic legacy POPs in the presence of the synthetic AR agonist methyltrienolone (R1881). *AR-tact Tasiilaq data; five male Inuit only. RLU, Relative light unit; Prt, Cell Protein; AhR-TEQ, aryl hydrocarbon receptor–TCDD toxic equivalent of serum extracts of lipophilic POPs; Itt, Ittoqqortoormiit; Tas, Tasiilaq; Nar, Narsaq; Nu, Nuuk; Sis, Sisimiut; Qeq, Qeqertarsuaq; Qaa, Qaannaq.

(DDE)] and anti-androgenic effects (e.g. DDE) [29]. Furthermore, additive enhancement of hormone actions has been reported *in vitro* for xenoestrogen and xenoantiandrogen mixtures ([41–43] and also *in vivo* for anti-androgens [44].

Human beings are exposed to a complex mixture of chemicals from various sources. As a result of the different and often opposed directed biological effects and potentials of the POPs, it is very difficult, if not impossible, to predict a given biological effect of the very complex mixtures of POPs that actually exist in the human body (e.g. blood). Therefore, the assessment of the combined biological effect of the actual chemical mixture in the human body is important. An approach to assess the combined mixture effects has been approximated by the extraction of the lipophilic POPs free of endogenous hormones from human serum followed by determination of the combined xenohormone and dioxin-like bioactivities of the extract *ex vivo* [36,45–51]. An array of *ex vivo* mammalian cell culture systems has been introduced to assess the combined xenohormone and dioxin-like induced bioactivities in human adipose tissue [52–54] and human serum [29,36,45–50]. The final combined effect reflects the integrated agonistic, additive or antagonistic mechanisms of the actual lipophilic POP serum mixture that may account as a mimic of the final *in vivo* effect. These studies have documented the relation between POP exposure and POP-related biomarkers of effects that can be used as an early effect biomarker of disease risk.

As for POP exposure biomarkers, studies on biomarkers of receptor effects in Greenland have shown district and gender differences across districts (fig. 2). A general inverse relationship between higher serum legacy POP concentrations and ER, AR and AhR transactivity was found. Accordingly, a higher frequency of serum samples with antagonistic ER and AR effects was observed for both sexes at the east coast

(Ittoqqortoormiit, Tasiilaq) and north-west (Qaanaaq), whereas higher frequencies of serum samples with agonistic ER and AR effects were observed for both sexes at the west coast (Qeqertarsuaq, Narsaq, Nuke, Sisimiut). However, for men in the two West Greenlandic districts, Nuuk and Sisimiut, a tendency towards increased serum POP-induced AR activity was observed (fig. 2) [29,36].

Performed with another method for serum POP extraction, including dioxin-like compounds, more than 75% of the serum POP extracts from both sexes elicited AhR-mediated dioxin-like activities. As for hormone receptor transactivities, the tendency was the higher serum legacy POP levels the lower AhR transactivities (fig. 2). The lowest median of the AhR-TCDD toxic equivalence (AhR-TEQ) values were observed in Ittoqqortoormiit and north-west (Qaanaaq), with higher AhR-TEQ levels for both sexes observed in (Tasiilaq, only five individuals), Narsaq, Sisimiut, Nuuk, and highest in the Disko Bay (Qeqertarsuaq) (fig. 2) [29,36]. A tendency to an inverse relation between the dioxin-like AhR and ER activity supports the perception that dioxins exert an anti-oestrogenic effect. We conclude that the actual mixtures of serum POPs in Greenlandic Inuit have a hormone-disrupting potential.

Interestingly, performed with the same method set-up, similar data for ER and AhR transactivity were observed in whole blood from East Greenlandic polar bears. However, compared to Inuit, higher frequency of agonistic xenohormone activity was seen in polar bears. We suggest that differences in metabolism of POPs causing higher levels of OH-PCBs circulating in the blood of polar bears might be part of the explanation but further investigation is needed. Although similar frequency of agonism towards the AhR, higher AhR-TEQ levels were found in polar bears that might be explained by an overall higher POP burden influenced by differences in the mixture profile and ratio of non-DL-PCB and DL-PCB compared to Inuit [55].

In a comparison between Inuit and young Danish women, the POP levels in Inuit were more than 10 times higher than the levels found in the Danes. Moreover, the levels were positively associated with age for both study groups [56]. The AhR-TEQ level differed between Inuit and Danish plasma samples, being significantly higher in Inuit, and AhR-TEQ were positively associated with plasma POPs, whereas no correlations were found for the Danish samples [56]. A recent study on AhR-TEQ in Danish individuals showed a higher level of AhR-TEQ for individuals living in urban areas compared to rural areas [57] indicating a higher exposure to dioxin-like compounds via food intake and traffic in urban areas.

Comparisons between European and Greenlandic male serum POP levels showed significantly higher levels in Inuit, and accordingly lower ER and AhR transactivity and a tendency towards higher AR activity for Greenlandic serum samples in relation to the European samples. However, in the same study, Inuit had significantly lower sperm DNA damage [29]. Further studies are required to elucidate whether the serum POP-related effects on sex hormone receptors and/or AhR are explanatory factors. Along with the intake of the traditional Greenlandic diet that contains high POP levels, there are also a number of important nutrients, such as trace elements/antioxidants and marine unsaturated fatty acids (e.g. omega 3) which have favourable effects on health, this issue constitutes 'The Arctic dilemma' [29]. A number of studies suggest that an increase in Western food items in the traditional diet of Inuit can lead to other health risks, such as the metabolic syndrome and its sequels increase in weight, hypertension, diabetes type 2, cardiovascular disease and cancer, including breast cancer (BC) [58].

To elucidate these aspects, further studies are required, including investigating biomarkers for exposure and effects, epigenetic contexts and the determination of relevant genetic polymorphisms, case–control as well as generation studies.

Epidemiological studies on biomarkers of POP exposure, effects, gene polymorphism and carcinogenicity.

Based on epidemiological studies, approximately 80% of all cancer is suspected be related to environmental exposures. Recently, PCBs and polybrominated biphenyls (PBBs) were classified as carcinogenic by IARC to groups 1 and 2 respectively [59]. Cancer sensitivity may be a result of differences in the genetic background of metabolism, DNA repair and changes in gene expression of tumour-related genes [28,29,60]. Therefore, gene polymorphisms in, for example, metabolizing enzymes such as the cytochrome P450 are suspected to influence sensitivity to environmental carcinogens. There are studies that have indicated coherence between gene polymorphisms in P4501A genes, the level of POPs and the risk for development of e.g. BC in Caucasians [28,29]. In a recent study, we showed that genetic polymorphisms differed significantly between Inuit and Europeans in the P450 phase I *CYP1A1* and *CYP1B1* genes and the phase II catechol-O-methyltransferase (*COMT*) gene [61]. For Inuit, the genotype distribution was more similar to those reported for Asian populations. Moreover, a significant difference between Inuit and Europeans in serum PCB153 and the *p*,*p*′-DDE levels, and for Inuit also associations between the POP levels and genotypes for *CYP1A1*, *CYP1B1* and *COMT* were found. These data provide new information on gene polymorphisms in Greenlandic Inuit which might support evaluation of susceptibility to environmental contaminants but warrant further studies.

Breast cancer is the most common cancer for women in the western world. From very few cases, an extraordinary increase in BC was observed in the Inuit population of Greenland and Canada during the last five decades, although still lower than in western populations [62]. Previous data suggest that exposure to POPs might contribute to the risk of BC. In a small case–control study in Greenlandic Inuit, a significant association between PFAA serum levels and high concentration of PCBs and BC risk was observed. Moreover, the data showed a higher frequency of cases with significant legacy POP-related hormone-like agonistic ER and AR effects, whereas AhR-TEQ equivalents were lowest in cases. This study showed for the very first time that serum POP levels, particularly PFAAs, might be risk factors in the development of BC

in Inuit. Furthermore, the results indicated that hormone disruption, as shown by the combined serum legacy POP-related xenoestrogenic and xenoandrogenic activities, may contribute to the risk of developing BC in Inuit [63].

We evaluated the association of BC risk with polymorphisms in genes involved in oestrogen biosynthesis, *CYP17* and *CYP19*, oestrogen metabolism and excretion, *CYP1A1*, *CYP1B1* and *COMT*. Imbalance between the phase I genes, *CYP1A1* and *CYP1B1*, involved in metabolism of environmental chemicals and oestrogens, and the phase II gene *COMT* that is involved in the clearance of produced hydroxylated catecholestrogen metabolites via the methylation pathway might increase the risk of BC (fig. 3). In the mentioned Inuit BC case–control study, we found as expected a higher frequency of the Greenlandic *BRCA1* founder mutation among cases and an independent association of *CYP1A1* (Val) and *CYP17* (A1) polymorphisms with BC risk, and the risk increased with higher serum levels of PFOS and PFOA [64]. Thus, the study indicates that serum PFAA levels are risk factors of BC in the Inuit, and inter-individual polymorphic differences might cause variations in sensitivity to the PFAA/POP exposure.

Epigenetic changes are a new paradigm in toxicology. It is a phenomenon that allows inheritable transfer of acquired DNA modifications without any changes to the primary DNA sequence. It is reflected by changes in the control of gene activity which originate from the interplay between DNA methylation, histone modification and RNA-mediated pathways. Epigenetic regulation is a part of the normal development and differentiation, but by disruption it may cause diseases such as cancer [28,29]. Exposure to, for example, PCBs and PBBs can play a role in two-stage cancer models involving DNA methylation. A small Greenlandic study including different districts showed a strong positive correlation between legacy POP serum concentrations and global hypomethylation of DNA [65], suspected to be involved in cancer risks. Further studies are required in order to elucidate these cellular and biological effects in relation to health.

Biomonitoring of Exposure and Effect Biomarkers of PFAAs

The PFAAs include perfluorinated carboxylic acids and the perfluorinated sulphonic acids. Among these groups, the two most studied PFAAs, PFOA and PFOS, are found. These two compounds are the most studied because existing laboratory procedures in the past did not allow analyses of other PFAAs that in general exist at lower concentrations. PFOA and PFOS are persistent in the environment and found in human blood, breast milk and liver with half-lives of 4–10 years [23]. The PFAAs are found globally, and governmental regulations in USA and Europe on use and production of specific compounds such as PFOS and PFOA have been made. Recently, PFOS was added to Annex B of the Stockholm Convention on POPs [17].

Biomonitoring studies have been carried out in almost all parts of the world in order to assess PFAA levels and temporal trends [66,67] and determinants [68] in the general population. European studies observed serum and plasma concentrations ranging from 1 to 116 ng/ml for PFOS and from 0.5 to 40 ng/ml for PFOA. The average plasma levels of PFOS and PFOA in Danish pregnant women were 35.3 and 5.6 ng/ml respectively, which are similar to most levels reported for populations of western countries during the same decade [69]. For middle-aged women in Norway, slightly lower levels were reported (medians: PFOS, 20 ng/ml; PFOA,

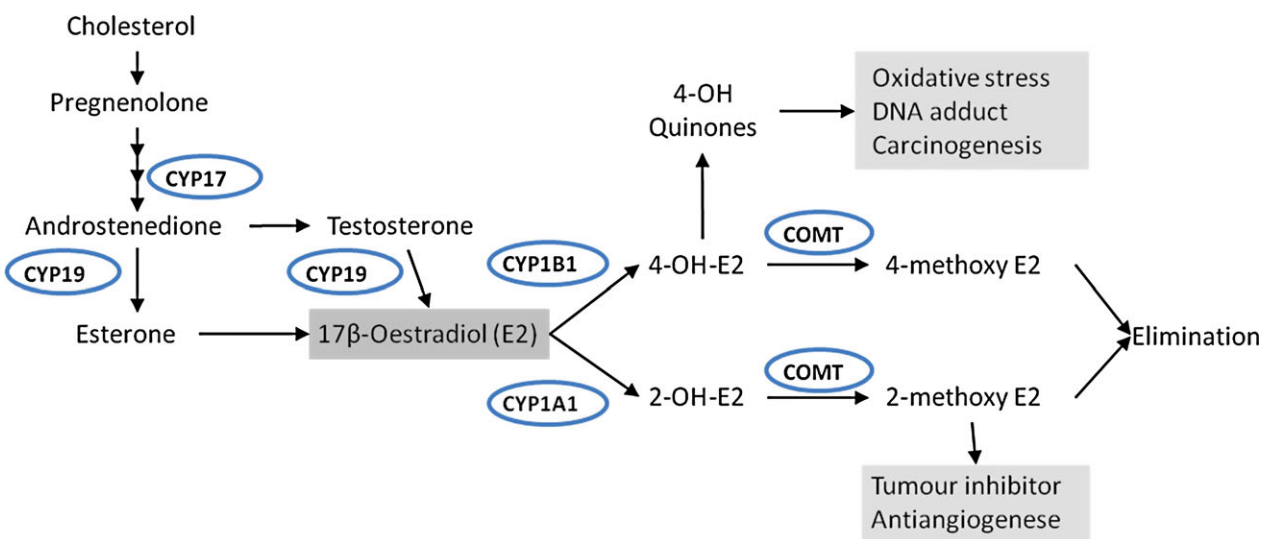

Fig. 3. Simplified schematic presentation of enzymes involved in oestrogen biosynthesis and metabolism [77]. CYP17 and CYP19 are involved in oestrogen biosynthesis. CYP1A1 and CYP1B1 are also involved in metabolism of environmental chemicals. 17β-oestradiol (E2) can be metabolized into the hydroxylated catecholestrogens, 2-OHE_2 and 4-OHE_2 by CYP1A1 and CYP1B1 respectively. These catecholestrogens can be cleared via the methylation pathway catalysed by COMT. Alternatively, the 4-OHE_2 can be oxidized into the semiquinone, which can undergo non-enzymatic conversion to its quinone generating superoxide radical.

4.4 ng/ml) [70]. Mean and median concentrations from North American populations appear to be slightly higher than European, Asian and Australian populations [67]. The maternal level of PFOA or PFOS was found associated with various reproductive and child health outcomes [69,71–74] and seems to impact maternal fecundity [72].

In a survey of PFAAs in Greenlandic Inuit [75], we found similar PFOS serum levels in Inuit women as found for Danish and European women, but lower PFOA levels. Recently, as mentioned in Biomonitoring of Legacy POPs in the Arctic, we reported for the very first time a significant association between PFAA serum levels and the risk of BC in Greenlandic women [63]. Moreover, we found that the genetic polymorphisms in *CYP1A1* (Val) and *CYP17* (A1) can increase the BC risk among Inuit women, and that the risk increases with higher serum levels of PFOS and PFOA [76].

Although less strong data, a current larger prospective epidemiological study on the BC risk in pregnant nulliparous Danish women [Bonefeld-Jørgensen EC, Long M, Fredslund SO, Bossi R, Olsen J, Submitted] supported the observed relation between BC risk and PFAA exposure as observed in the Greenlandic Inuit women [63].

Biomarker of PFAA effects ex vivo *studies in human beings.*
The assessment of the combined biological effect of the actual chemical mixture in the human body has been approximated by extraction of the lipophilic legacy serum POPs followed by measurement of the combined xenohormone and dioxin-like bioactivities of the extract *ex vivo* (see Biomarkers of hormone-disruptive effects of legacy POPs *ex vivo*). To approach the assessment of both the combined mixture effect of the legacy serum POPs and the combined mixture effects of the serum PFAAs, we have for the very first time developed a method for extraction of PFAAs from human serum with simultaneous removal of endogenous sex hormones. Analysis of the extracted PFAA serum fraction from pregnant women documented that the fractions were free of ER-active endogenous hormones. Furthermore, we found that the actual combined mixture of serum PFAAs had the potential to significantly induce ER transactivation when tested alone, and further increase the 17β-oestradiol-induced ER transactivity upon co-exposure with this highly potent ER ligand. Thus, our data suggest that the PFAAs – at levels found in human serum – may play a role in endocrine disruption via the ER [Bjerregaard-Olesen C, Bossi R, Bech BH, Bonefeld-Jørgensen EC, Submitted].

Biomarker of PFAA effect studied in rodents.
The biological effects of PFAAs have been studied in more detail mainly in rodents; few data are available for other species and human beings [25,77]. Studies in animals have documented an array of toxicological outcomes including liver hypertrophy and tumours [78], TH alterations, decreased serum cholesterol and glucose, developmental toxicity, immunotoxicity and carcinogenic potency [79,80]. Animal and *in vitro* studies have also suggested that PFAAs may have potential geno- and neurotoxic effects [81,82]. The U.S. EPA has proposed PFOA to be deemed as a rodent carcinogen with relevance to human beings [83]. A two-year study in rats [84] reported a statistically significant increase in mammary fibroadenomas and Leydig cell adenomas, suggesting an impact of PFOA on reproductive tissues, whereas two other rat studies did not find increased incidence of mammary-gland neoplasms upon a 2-year chronic dietary administration of ammonium perfluorooctanoate [85,86]. Thus, conflicting data for PFOA exposure in rats are reported. In mice, however, gestational exposure to PFOA compared to non-exposed controls was found associated with altered mammary gland development in dams and female offspring, and a significant reduction in mammary differentiation among exposed dams was evident also affecting the epithelial involution and altered milk protein gene expression [87]. Because of these data, the U.S. EPA Science Advisory Board recommended to reconsider the possible impact of PFOA on mammary tissues [83,88].

Biomarker of PFAA effect studied in vitro.
In vitro studies have demonstrated endocrine-disrupting (ED) potentials of the PFAAs. Oestrogenic properties of PFAAs were reported in human MCF-7 BC cells [89]. Recently, we demonstrated in mammalian cell culture models the ED potential of five of seven tested PFAAs (PFOS, PFOA, PFHxS, PFNA, PFDA, PFUnA, PFDoA) [8]. Three PFAA elicited agonistic effects on ER transactivity (PFOS, PFOA, PFHxS) and five antagonistic effects on AR transactivity (PFOS, PFOA, PFHxS, PFNA, PFDA) indicating additive combined mixture effects (fig. 4). PFDA also weakly decreased the aromatase activity at a high test concentration [8]. The seven tested PFAAs also affected the TH function by inhibiting the rat pituitary GH3 cell growth (fig. 5); four of the PFAAs (PFOS, PFHxS, PFNA and PFUnA) also antagonized the T3-induced GH3 cell growth [90]. Only PFDoA and PFDA elicited an activating effect on the AhR transactivation [90]. Moreover, fluorochemicals and their metabolites present in food packaging materials were reported to affect steroidgenesis in H295R human adrenal cortico-carcinoma cells, decreasing and increasing gene expression of Bzrp and CYP19, respectively, leading to lower androgen and higher oestrogen levels [6].

The PFAAs are suspected carcinogens and a possible mechanism of action is generation of oxidative stress.

In our recent *in vitro* study performed with human liver HepG2 cells, we observed a dose-dependent DNA damage induced by PFOS, PFOA, PFHxS and PFNA in a non-cytotoxic concentration range, and significant reactive oxygen species (ROS) induction by PFOS, PFOA, PFHxS, PFDA and PFNA although not dose-dependently. Moreover, PFOA showed the potential to decrease the total antioxidant capacity [Wielsøe M, Long M, Ghisari M, Bonefeld-Jørgensen EC, Submitted].

In contrast, Eriksen *et al.* [91] found no DNA damage upon exposure of HepG2 to PFOS and PFOA, whereas in support of our study a modest increase in DNA damage by PFNA and exposure to PFOS and PFOA generated a non-concentration dependent ROS. Another study also performed withHepG2 cells, supported the Eriksen *et al.* study, showing that PFOA

A Oestrogen receptor transactivity in human breast carcinoma (MVLN) cells

PFAAs tested with 25 pM E2	LOEC (M)	MOEC (M)	% of solvent + 25 pM E2 ± SD
Solvent			100
PFHxS	$4x10^{-5}$	$9x10^{-5}$	187±24
PFOS	$1x10^{-5}$	$5x10^{-5}$	210±13
PFOA	$3x10^{-5}$	$9x10^{-5}$	145±15
Mix	$3.5x10^{-5}$	$3.5x10^{-5}$	150±25

B Androgen receptor transactivity in Chinese hamster ovary (CHO-K1) cells

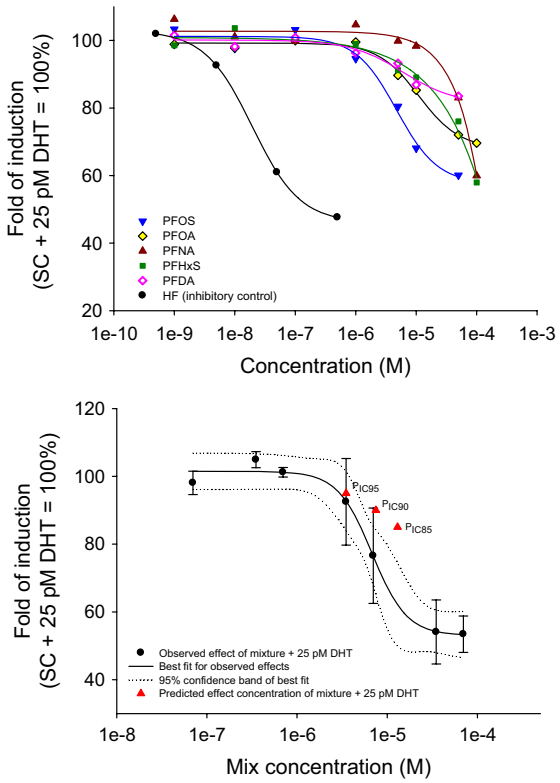

Fig. 4. Oestrogenic and anti-androgenic effects of perfluorinated alkyl acids (PFAAs) in mammalian cell cultures [8]. Seven PFAAs (PFOS, PFOA, PFHxS, PFNA, PFDA, PFUnA, PFDoA) were tested as individual compounds and as an equimolar mixture in sex hormone receptor transactivation assays. (A) Oestrogen receptor transactivity in human breast carcinoma (MVLN) cells: The seven PFAAs were tested as individual compounds and in mixture upon co-exposure with 25 pM of the natural oestrogen receptor ligand 17β-oestradiol (E2) to mimic possible *in vivo* interactions between the test compounds and a natural oestrogen hormone. LOEC: the lowest tested concentration at which a significant effect ($p < 0.05$) was detected; MOEC: the lowest tested concentration causing the maximum effect (non-toxic); S.D.: standard deviation. No effects were observed for PFNA, PFDA, PFUnA and PFDoA when these compounds were tested alone (data not shown). (B) Androgen (AR) receptor transactivity in Chinese hamster ovary (CHO-K1) cells: The seven PFAAs were tested as individual compounds and in mixture upon co-exposure with 25 pM of the natural androgen receptor ligand dihydrotestosterone (DHT) to mimic possible *in vivo* interactions between the test compounds and a natural androgen hormone. No effects were observed for PFUnA and PFDoA when these compounds were tested alone (data not shown). Combined effects of the mixture were assessed performed with the principle of concentration addition (CA). HF, hydroxyflutamide (inhibitory control); SC, solvent control; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate.

and PFOS exerted a cytotoxic effect, but no increase of DNA damage or ROS [92]. In contrast, Yao and Zhong [93] reported a PFOA-induced dose-dependent DNA damage and significantly increased ROS in HepG2 cells. Furthermore, using non-tumour hepatic cells (L-02), analyses revealed that PFOA induced oxidative stress, cell cycle arrest and apoptosis, and a proteomic study proposed that PFOA induced stress via inhibition of some proteins (GRP78, HSP27, CTSD, hnRNPC) which may be involved in the activation of p53 and induced apoptosis [94].

In summary, these to some degree controversial *in vitro* studies suggest that some of the PFAAs have the potential to induce oxidative stress in terms of ROS production and DNA damage in the cell line representing the human liver; however, further studies are needed.

Conclusion and Perspectives

An array of lipophilic legacy POPs, including PCDD/PCDF, PCBs, OCPs and the amphiphillic PFAAs, are potential

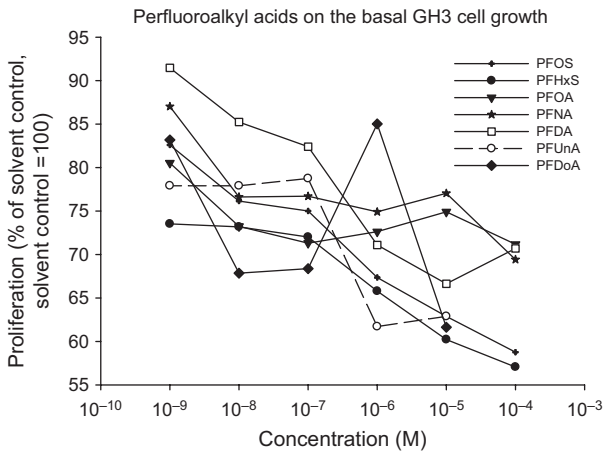

Fig. 5. Effects of perfluoroalkyl acids on the basal GH3 cell growth in the T-screen assay [90]. GH3 cells were incubated with the given concentration (M) of compounds in the absence of 3,3′,5-Tri-iodo-L-thyronine (T3). Data represent mean of at least three independent experiments, each performed in four replicated per concentration. Exposure to the seven PFAAs (PFOS, PFOA, PFHxS, PFNA, PFDA, PFUnA, PFDoA) all significantly decreased the cell proliferation compared to the corresponding solvent control (0.02% dimethyl sulphoxide or 0.02% ethanol) ($p < 0.05$). The results given refer only to effects observed at non-cytotoxic concentrations. PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; PFAA, perfluorinated alkyl acid.

endocrine disrupters, have carcinogenic potentials, and can play an important role in health risk. These environmental persistent compounds do biomagnify up through the food web and bioaccumulate in human beings and animals. Legacy POPs as well as PFAAs have been associated with effects being relevant for development of hormonal disruptive disease having xenoestrogenic, xenoandrogenic, tumour promoting and immunosuppressive activities. Biomonitoring studies on serum POP exposures and biomarkers of effects *ex vivo* of the combined serum POP mixture have the potential to elucidate the relationship between emissions, exposure, biological effects and health risks. Determination of relevant genetic polymorphisms might help to assess the health risk of an individual upon exposure to POPs. Further epidemiological molecular-genetic studies are warranted to document the effect observed *ex vivo* for human beings as well as studies in animals and *in vitro*.

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