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REVIEW

Receptor research on xenohormone effects of human serum extracts containing the actual mixture of perfluorinated alkyl acids: a short review

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Perfluorinated alkyl acids (PFAAs) are used in many household products including food contact materials. Hence, humans are continuously exposed, and the PFAAs are accumulated in human serum with half-lives up to 8.8 years. In humans, high PFAA serum levels have been associated with an increased risk of breast cancer and other adverse health effects such as lower birth weight and longer time to pregnancy which might be related to disruptions of various hormonal systems. For instance, direct cell exposure studies *in vitro* suggest that some PFAAs can transactivate the estrogen receptor (ER), antagonize the androgen receptor (AR) and has the potential to interfere with Thyroid Hormone and Aryl-hydrocarbon Receptor functions. Moreover, the PFAAs also showed cellular oxidative stress potential. Humans are exposed to an array of PFAAs, and the quantity and combination of these PFAAs in human serum differs between individuals. Hence, the toxicological studies of single PFAAs and simple mixtures might be insufficient to predict how the actual mixtures of PFAAs may affect humans. To get a better evaluation of the actual mixture effects, we developed a method to extract the actual mixture of PFAAs from human serum. Preliminary results showed that 17% of the PFAA serum fractions from pregnant women could significantly transactivate the ER, and 94% of the fractions could further increase the transactivity induced by the potent ER ligand 25 pM 17β-estradiol. As part of the international FETOTOX project (http://fetotox.au.dk/), we are currently extracting the actual PFAA serum mixture from 700 pregnant women to further elucidate the potential of the serum PFAA mixture to transactivate the ER at the levels found in human serum. We suggest that our method can in the future be used to study the effects of the actual serum PFAA mixture on both steroid hormone actions as well as other hormonal systems e.g. thyroid hormone function. In the current review we will discuss how our recently developed PFAA extraction method might be used in future research to assess the endocrine impact of PFAAs on human health.

Keywords: Androgen receptor; Estrogen receptor; Extraction method; Mixture effects; Perfluorinated compounds; Persistent Organic Pollutants

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Per- and polyfluorinated alkyl substances (PFASs) are a group of manmade compounds that are used in many products e.g. coating formulations, fire-fighting foams, insecticides, polyurethane production, inks, varnishes, lubricants, gasoline, and oil and water repellents for leather, textiles and paper including food contact materials [1]. A subgroup of these compounds - the *perfluorinated alkyl acids* (PFAAs) - will be the main focus of this research highlight.

The PFAAs contain a fully fluorinated alkyl chain and an anionic head group, usually a carboxylic acid or a sulfonic acid. Perfluorinated sulfonic acids with six or more carbon atoms and perfluorinated carboxylic acids with eight or more carbon atoms are defined as long-chain PFAAs by the US EPA [2]. The long-chain PFAAs are ubiquitous in the environment including drinking water [3] and bioaccumulate up through the food web and in human beings [4]. Furthermore, the long-chain PFAAs have long half-lives in human serum up to 3.8 - 8.8 years for perfluorohexanoic sulfonate (PFHxS), perfluorooctanoic sulfonate (PFOS) and perfluorooctanoic acid (PFOA) [5], whereas the short-chain PFAAs are suggested to be more easily excreted [4].

Studies have shown that PFAAs can be transferred from mother to fetus via the cord blood [6, 7]. Furthermore, several PFAAs have also been detected in breast milk although at much lower concentrations compared to human serum [4, 6, 7]. Thus, exposure to PFAAs is happening during the early and very vulnerable stages of life. This exposure might lead to some adverse health effects at birth as well as later in life [8]. For instance, Fei *et al*. found that maternal serum levels of PFOA were inversely associated with birth weight among newborns from the Danish national birth cohort [9]. This was supported by Maisonet *et al*. who found that British girls born to mothers with PFOA, PFOS and PFHxS concentrations in the upper tertile on average weighed less than those in the low tertile [10]. However, 20 months after the birth, the girls born to mothers with PFOS concentrations in the upper tertile weighed significantly more than those in the lowest tertile. This accelerated growth of the newborn baby was suggested to be a compensation for the restrained intrauterine growth. This hypothesis was further substantiated by Halldorsson *et al*. who reported that maternal PFOA concentrations during pregnancy were positively associated with BMI and waist circumference among the female offspring at 20 years of age [11]. Thus, the PFAA exposure during intrauterine development and infancy might also interfere with adult health. Furthermore, Vested *et al*. reported that *in utero* PFOA exposure may affect the semen quality in adult men, which was observed as lower sperm concentration and a lower total sperm count [12].

High PFAA levels have also been associated with delayed puberty [13], longer menstrual cycles [14], reduced fertility [15], and breast cancer [16, 17] amongst others. To elaborate, Lopez-Espinosa *et al*. found that boys in the highest PFOS quartile had a delayed onset of puberty of 190 days compared to boys in the lowest quartile. Furthermore, the puberty of girls in the highest PFOS and PFOA quartiles were on average postponed with 138 and 130 days, respectively, compared to girls in the lowest exposure group [13]. Recently, Lyngsø *et al*. found that high PFOA exposure was significantly associated with longer menstrual cycles, and both PFOA and PFOS were associated with increased odds of having irregular menstrual cycles although not significantly [14]. In addition, Fei *et al*. reported that the odds of infertility increased by 70-134% and 60-154% among women in the higher three quartiles when compared with the lowest quartiles of PFOS and PFOA, respectively [15]. Infertility was defined as having a time to pregnancy longer than 12 months or receiving treatment for infertility.

In 2000, the PFAS manufacturer 3M started phasing out the production of C6, C8 and C10 PFAAs including PFOS and PFOA [18]. The compounds are however still detected in human serum. For instance, in a currently ongoing study (http://fetotox.au.dk/) we analyzed sixteen PFAAs in the serum from approximately 1530 pregnant Danish women and found that more than 99 percent of these samples contained PFHxS, PFOS, PFOA, perfluorononanoic acid (PFNA), and perfluorodecanoic acid (PFDA) at levels above the limit of quantification [Bjerregaard-Olesen, *in preparation*]. Although many studies have shown that the serum PFAA levels have decreased since the phase-out [19-21], there have also been reports of increasing trends in some parts of the world. For instance, we found that PFOS significantly increased in Greenlandic Inuit women from Nuuk in the period 1998 – 2005 [22]. The levels of PFNA, PFDA, perfluorododecanoic acid (PFDoA), and perfluorotridecanoic acid (PFTrA) were also found to significantly increase when both men and women were included in the analysis. Upon adjustment for age, the trends were however no longer significant, though weak increases of PFOS, PFOA, PFNA, PFDA, and PFDoA were still observed [22]. A study from Shenyang, China, also showed a dramatic increase of PFOS and PFOA concentrations between 1999 and 2002 [23].

The International Agency for Research on Cancer (IARC) has classified PFOA as “possibly carcinogenic to humans” [24]. A potential carcinogenic mode of action is through the generation of oxidative stress, and we recently found that PFHxS, PFOA, PFOS and PFNA elicited a dose-dependent increase in DNA damage to human hepatoma cells [25].In the same study, PFHxS, PFOS, PFOA, PFNA, PFDA and perfluoroundecanoic acid (PFUnA) increased the generation of reactive oxygen species. In two recent studies involving female participants from Greenland and Denmark we found an increased risk of breast cancer with increasing serum levels of PFOS and perfluoroctanoic sulfonamide (PFOSA), respectively [16, 17]. This breast cancer risk was further increased for Greenlandic women with CYP1A1 Val, and/or CYP17A1 polymorphisms [26].

All of the mentioned adverse health effects might be caused by disruption of various hormonal systems e.g. the

estrogen receptor (ER), the androgen receptor (AR), the thyroid receptor and/or the aryl hydrocarbon receptor (AhR). Especially, the ER is considered to be quite promiscuous meaning that many different compounds have been shown to bind and interfere with the ER. By the use of the human breast cancer derived MVLN cell line carrying an estrogen response element - luciferase reporter vector, we found that PFOS, PFOA and PFHxS transactivated the ER in a dose-dependent manner [27], whereas PFNA, PFDA, PFUnA and PFDoA did not significantly transactivate the ER. However, an equimolar mixture containing PFHxS, PFOS, PFOA, PFNA, PFDA, PFUnA, and PFDoA significantly and additively transactivated the ER since the maximal effect was reached at a lower concentration for this mixture than the concentration needed for the individual PFAAs to transactivate the ER [27]. In contrast, five of these tested PFAAs - PFHxS, PFOS, PFOA, PFNA and PFDA - significantly antagonized the AR activity in a concentration dependent manner. Furthermore, a more than additive mixture effect was observed on the AR function. Moreover, PFDA weakly decreased the aromatase activity at a high test concentration [27].

This indicates that it is important to study the effects from exposure to mixtures of environmental compounds including the PFAAs. The industrial production of chemicals is ever changing and human exposure to these chemicals will therefore also be changing over time. Depending on the combination of compounds in the mixture, the actual biological effects may be enhanced or counteracted in comparison with the action of the single compounds alone [28]. Given the complexity of the human exposure and the various modes of action, it would be naive to believe that the real effects of these cocktails can be predicted by studying the effects of single chemicals alone or as simple mixtures.

A way to study the effects of the actual mixture of compounds relevant for the human body is by extracting the compounds from human tissues e.g. from human serum [29]. In 2006, we established a method to extract lipophilic persistent organic pollutants (POPs) including the polychlorinated biphenyls (PCBs) and organochlorine pesticides from human serum [30]. Following extraction, the effects of the real and actual serum POP mixture on the ER or AR functions can be studied as we have previously done in several studies using serum from Inuit as well as European populations [16, 31-36].

We recently developed a method extracting both the lipophilic POPs and the PFAAs, free of endogenous hormones, from the same serum sample [37] by expanding the method for extraction of the lipophilic POPs [30]. The method development included a new liquid/liquid extraction method, modification of the HPLC fractionation, and introduction of an anion exchange step. For documentation of the new method, we extracted the PFAA fraction from the serum of 18 pregnant Danish women and subsequently determined the effect of these PFAA serum fractions on the ER function using the stably transfected MVLN cell line [37]. The PFAA fraction from three of the 18 serum samples significantly transactivated the ER. When the MVLN cells were co-treated with the natural ER-ligand 17β-estradiol (E2), 17 of the 18 PFAA fractions caused a significant further increase of the E2 induced ER transactivity [37]. We therefore suggest that the actual mixture of PFAAs at the levels found in human serum can transactivate the human ER. To further test this hypothesis we are currently extracting the PFAAs from the serum of 700 Danish pregnant women as part of the international FETOTOX project (http://fetotox.au.dk/). Upon the extraction of the PFAAs from these serum samples we will determine the effect of the actual PFAA mixtures on the ER transactivation and evaluate whether the effects are related to the serum levels of PFAAs. Furthermore, we also plan to compare the PFAA induced ER transactivities with time to pregnancy as well as birth outcomes such as birth weight and head circumference.

The effect on the ER function has been tested separately for the PFAA and lipophilic POP serum fractions. We plan to also determine the combined effect of these two fractions. As mentioned, the PFAA fraction has been shown to exert an estrogenic effect [37], whereas the fraction of lipophilic POPs primarily exerts an antiestrogenic effect [29]. Therefore, the observed ER transactivities from the two fractions separately might counteract when combined. However, the chemical profile might affect the final interference with the hormone receptors, since in some study groups we have observed that the lipophilic POP fraction elicited an agonistic effect on the ER [35]. That might also suggest that in some individuals there might be a potential for synergistic effects between the PFAAs and lipophilic POPs on the ER.

The PFAA extraction method was initially validated by extraction of seven long-chain PFAAs (PFHxS, PFOS, PFOA, PFNA, PFDA, PFUnA and PFDoA) for which mean recoveries between 49.6% and 78.6% were obtained. Human serum however also contains several other PFASs. We expect that other long-chain PFAAs such as perfluoroheptanoic sulfonate (PFHpS) and PFTrA will be extracted with recoveries that are comparable to the seven initially tested PFAAs. However, the short-chain PFAAs and the uncharged PFASs such as PFOSA and the fluorotelomer alcohols must also be taken into consideration. We plan to determine the recoveries of these compounds and if necessary further modify the method such that more PFASs can be included while simultaneously removing endogenous

hormones such as estrone, estradiol and testosterone. It is important to remove these endogenous hormones before analyzing the receptor transactivities, as the effect of the high potency endogenous hormones will otherwise affect the sensitivity when analyzing the effect of the xenohormones.

We have also analyzed the lipophilic serum POP fractions' effects on the AR transactivity [29, 31-34, 38]. Recently, we further developed the method for determination of the AR transactivities of the serum PFAA fraction by inhibiting interfering endogenous androgen metabolites that seems to be formed during the cell culture AR transactivity analysis (Bjerregaard-Olesen C, Kjeldsen LS, Ghisari M, and Bonefeld-Jørgensen EC; *submitted*). *In vitro* direct cell exposure to PFHxS, PFOS, PFOA, PFNA and PFDA alone as well as in an equimolar mixture (also including PFUnA and PFDoA) elicited antiandrogenic responses [27]. Thus, we suggest that the actual serum mixture of PFAAs might have an antagonistic effect on the AR function, which we work on documenting in the near future.

Furthermore, we recently found that PFDA and PFDoA have the potential to transactivate the arylhydrocarbon receptor (AhR), a receptor involved in the synthesis of steroid hormones as well as metabolisms by inducing the transcription of e.g. the CYP1A1 and CYP1B1 genes [39]. We also found *in vitro* that PFHxS, PFOS, PFOA, PFNA, PFDA, PFUnA and PFDoA inhibited the growth of the thyroid hormone (TH) dependent GH3 cells upon direct cell exposure [39], and four of these (PFHxS, PFOS, PFNA and PFUnA) could also antagonize the 3,3',5-triiodo-L-thryonine (T3) induced GH3 cells proliferation upon co-exposure. The THs are essential for fetal growth and normal brain development, and thus the inhibition of TH functions might interfere with the development of the fetus. Adult health may however also be at risk. For instance, Melzer *et al*. have reported significant associations between PFOA and PFOS concentrations and treated thyroid disease in women and men, respectively [40]. The underlying mechanisms behind this association is however still not clear and more work is needed to elucidate the mechanisms. In Melzer *et al*.'s study they focused entirely on PFOS and PFOA but we suggest that the interactions with more PFAAs and possibly other compounds could be involved in the underlying mechanisms. Among the other compounds that may be involved are the hydroxylated PCBs (OH-PCBs), which can bind to the TH transport protein *transthyretin* that is involved in the transfer of THs across the placenta and blood-brain barrier [41-43]. Furthermore, OH-PCB 69, OH-PCB 106 and OH-PCB 121 have been shown to stimulate the proliferation of the TH-dependent GH3 cells *in vitro* [44]. We are currently testing whether our two methods for human serum extraction of the lipophilic POPs [30] and serum extraction of the PFAAs [37] can be modified to also include the extraction of OH-PCBs from the same human serum sample. We want to determine whether the serum OH-PCB fraction and the PFAA fraction can affect the proliferation of the TH-dependent GH3 cells separately and combined. This may be used as a means to explore whether these two groups of environmental compounds can elicit any synergistic or counteracting effects on the TH functions.

In conclusion, the lipophilic POPs as well as the PFAAs bioaccumulate in humans, and the compounds have been associated with adverse health effects that might be related to hormone disruption through e.g. estrogenic, androgenic and thyroid-interfering activities. We have recently established and documented a method for serum extraction of both the lipophilic POPs and the amphiphilic PFAAs, from the same serum sample, while simultaneously removing endogenous hormones such as estrone, estradiol and testosterone. By extracting the PFAA and the lipophilic POP fractions from human serum and subsequently analyzing the xenohormonal effects of the fractions, it is possible to elucidate the relationship between exposure to lipophilic POPs and PFAAs and associated biological effects and health outcomes. Currently we are working on further developing the extraction methods such that other compounds like the uncharged PFASs and the OH-PCBs can be extracted at the same time using the same serum sample. By combining the various fractions of environmental chemicals from human serum we expect to explore the actual xenohormonal effects for the separate chemical groups as well as the mixed cocktail effects.

Conflicting interests

The authors have declared that no competing interests exist.

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Abbreviations

AhR:Arylhydrocarbon receptor; AR: Androgen receptor; E2: 17β-estradiol; ER: Estrogen receptor; OH-PCB: Hydroxylated polychlorinated biphenyl; PCB: Polychlorinated biphenyl; PFAA: Perfluorinated Alkyl Acids; PFAS: Per- and polyfluorinated alkyl substances; PFDA:

Perfluorodecanoic acid; PFDoA: Perfluorododecanoic acid; PFHpS: Perfluoroheptanoic sulfonate; PFHxS: Perfluorohexanoic sulfonate; PFNA: Perfluorononanoic acid; PFOA: Perfluorooctanoic acid; PFOS: Perfluorooctanoic sulfonate; PFOSA: Perfluoroctanoic sulfonamide; PFTrA: Perfluorotridecanoic acid; PFUnA: Perfluoroundecanoic acid; POP: Persistent organic pollutant; T3: 3,3',5-triiodo-L-thryonine; TH: Thyroid Hormone.

Contributions

Both authors contributed to the design of the work, CBO wrote the first draft of the paper and EBJ critically revised the paper, both authors read and approved the final version.

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