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Review article

Receptor-based *in vitro* activities to assess human exposure to chemical mixtures and related health impacts

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ABSTRACT

Humans are exposed to a large number of chemicals from sources such as the environment, food, and consumer products. There is growing concern that human exposure to chemical mixtures, especially during critical periods of development, increases the risk of adverse health effects in newborns or later in life. Historically, the one-chemical-at-a-time approach has been applied both for exposure assessment and hazard characterisation, leading to insufficient knowledge about human health effects caused by exposure to mixtures of chemicals that have the same target. To circumvent this challenge researchers can apply *in vitro* assays to analyse both exposure to and human health effects of chemical mixtures in biological samples. The advantages of using *in vitro* assays are: (i) that an integrated effect is measured, taking combined mixture effects into account and (ii) that *in vitro* assays can reduce complexity in identification of Chemicals of Emerging Concern (CECs) in human tissues.

We have reviewed the state-of-the-art on the use of receptor-based *in vitro* assays to assess human exposure to chemical mixtures and related health impacts. A total of 43 studies were identified, in which endpoints for the arylhydrocarbon receptor (AhR), the estrogen receptor (ER), and the androgen receptor (AR) were used. The majority of studies reported biological activities that could be associated with breast cancer incidence, male reproductive health effects, developmental toxicities, human demographic characteristics or lifestyle factors such as dietary patterns. A few studies used the bioactivities to check the coverage of the chemical analyses of the human samples, whereas *in vitro* assays have so far not regularly been used for identifying CECs in human samples, but rather in environmental matrices or food packaging materials.

A huge field of novel applications using receptor-based *in vitro* assays for mixture toxicity assessment on human samples and effect-directed analysis (EDA) using high resolution mass spectrometry (HRMS) for identification of toxic compounds waits for exploration. In the future this could lead to a paradigm shift in the way we unravel adverse human health effects caused by chemical mixtures.

1. Introduction

Humans are exposed to multiple chemicals simultaneously from sources such as the environment (air, dust, water), food and food packaging, and consumer products.. There is growing concern that exposure to mixtures of environmental chemicals affects human health emphasizing the relevance and necessity of thorough studies of associations between chemical exposures and human health outcomes (Kortenkamp, 2007; Kortenkamp, 2009; Svingen and Vinggaard, 2016; http

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Nomenclature

Abbreviations

ADI	Acceptable daily intake
AhR	Arylhydrocarbon receptor
AR	Androgen receptor
CEC	Chemical of Emerging Concern
EBT	Effect-based trigger value
EDA	Effect-directed analysis
ER	Estrogen receptor
FCM	Food contact material
HBM4EU	Human Biomonitoring for Europe (EU Joint program 2017–21)
HRMS	High resolution mass spectrometry
NORMAN	Network of reference laboratories, research centres and related organisations for monitoring of emerging environmental substances
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PFAS	Per- and polyfluoroalkyl substances
POP	Persistent organic pollutants
PPARα/ϒ	Peroxisome proliferator-activated receptor α/ϒ
T4	Thyroxine
2,3,7,8-TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TEQ	2,3,7,8-TCDD equivalent
TDI	Tolerable daily intake
TTR	Transthyretin

Definitions

Effect biomarker A measurable biochemical, physiological, behavioral, or other alteration in an organism that, depending on the magnitude, can be recognized as associated with an established or possible health impairment or disease (NRC, 1987)

Exposure biomarker A chemical, its metabolite, or the product of an interaction between a chemical and some target molecule or cell that is measured in the human body (NRC, 1987)

s://youtu.be/Gp4zS5CmX-E). Researchers, however, have primarily studied exposure to each chemical seperately. This one-chemical-at-a-time approach has left us with insufficient knowledge about the adverse human health effects caused by exposure to chemical mixtures. Thus, for assessment of adverse human health effects, the association of combined biological effects with identification and quantification of causative chemicals, especially in biological tissues, is warranted for obtaining a better understanding of environmental impact on human disease.

1.1. Mixture effects of chemicals

Evidence from the last decades has shown that adverse effects of the combined exposure to environmental chemicals have to be taken into account in chemical risk assessment (Boberg et al., 2019; Howdeshell et al., 2017; Svingen and Vinggaard, 2016). Experimental studies on model compounds, which have been designed to investigate potential interactions between chemicals that affect the same targets, indicate that chemicals in most cases are acting additively at low realistic dose levels. This means that it is possible by mathematical modeling to predict the mixture effect by concentration addition (Kortenkamp, 2009). In concentration addition, the mixture toxicity is predicted by summing up the contribution of all individual compounds based on their individual toxic potency and their concentration in the mixture. As it is neither feasible nor productive to test every imaginable chemical mixture, this is of significant importance for risk assessment, because this means we can estimate the mixture effect based on knowledge of hazard and exposure data of single chemicals.

An additional approach has been to study mixtures of large number of chemicals defined from human biomonitoring data and to test them in animals or cell cultures. Examples include investigations of environmentally relevant mixtures of persistent organic pollutants (POPs) and/or pesticides at relevant dose levels, and these studies have indicated that the current human exposure to chemical mixtures may leave a pathophysiological footprint on the intact organism, thereby affecting human health (Ahmed et al., 2019; Berntsen et al., 2017; Hadrup et al., 2016; Martin et al., 2017).

Presently, several theoretical case studies are ongoing in the European H2020 joint program HBM4EU (Human BioMonitoring for Europe; https://www.hbm4eu.eu/) that aims to predict effects caused by exposure to chemical mixtures such as known antiandrogenic chemicals on human male reproductive health and known neurotoxic chemicals on human brain development. In these case studies, the predictions are based on existing knowledge on average human chemical exposures determined by classical human biomonitoring and by hazard information obtained from both *in vivo* or *in vitro* data.

The drawback of the approaches is that the predictions are limited to chemicals with known effects and known presence in human tissues. As we do not have the full picture of the human exposome and of the hazards that these chemicals may cause, these approaches are likely to underestimate the human risk. Therefore, new and integrative experimental approaches are needed to elucidate the link between human chemical mixture exposure and health effects.

1.2. The traditional way of detecting biomarkers of exposure in humans

The traditional way to detect human biomarkers of exposure is to use a bottom-up approach (Fig. 1A) in which statistical assessment of associations between specific chemical exposure derived from human biomonitoring and health endpoints are performed in epidemiological studies. Relatively few chemicals with known or suspected toxicological properties are usually selected for human biomonitoring analyses. In some cases the bottom-up approach will result in relatively good evidence of a link between a specific chemical exposure and disease outcome, as illustrated for confirmed adverse effects caused by e.g. persistent organic pollutants (POPs) (Govarts et al., 2012; Krall et al., 2015; Liew et al., 2018; Radke et al., 2018). In other cases, results will vary from study to study, and no firm conclusions can be drawn (Bonde et al., 2016; Magliano et al., 2014; Mouly and Toms, 2016).

The bottom-up approach applying targetted analysis of human samples would usually not be an appropriate tool for revealing exposure to all chemicals that may be associated with a specific disease outcome, as the integrated biological activity (the mixture effect) of all relevant chemicals in the human body is not taken into account, adding uncertainty to the outcome. Thus, traditional chemical-based risk assessment often fails to explain the hazard contributions from the mixture of environmental chemicals that are not regularly monitored or identified yet.

1.3. The traditional way of detecting biomarkers of effect in humans

Effect biomarkers, defined as '*a measurable biochemical, physiological, behavioral, or other alteration in an organism that, depending on the magnitude, can be recognized as associated with an established or possible health impairment or disease*' (NRC, 1987), are routinely measured in human blood or urine samples (e.g. glucose, cholesterol, C-reactive

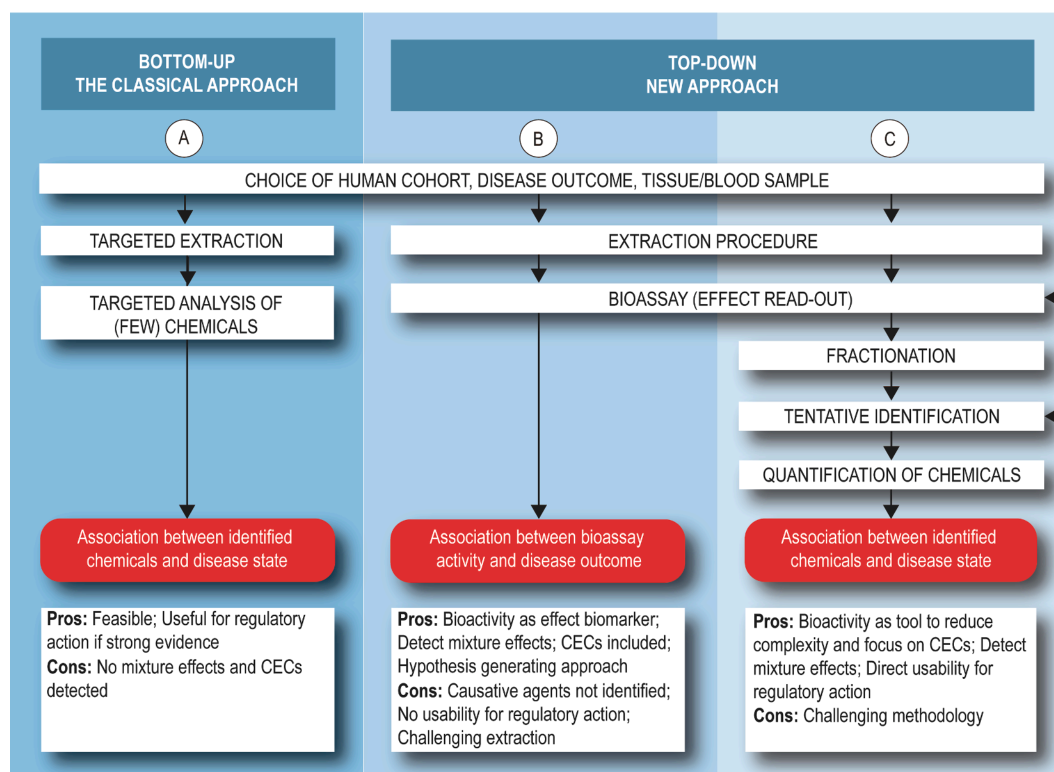

Fig. 1. Various approaches for associating chemical exposures to disease outcomes. (A) Illustration of the classical 'bottom-up' approach in which targeted chemical analysis is performed on human tissues and associated with a health endpoint. (B) Illustration of the approach where a specific *in vitro* activity caused by the chemicals defined by the extraction procedure is associated with a health outcome without identifying the specific responsible chemicals. (C) Illustration of EDA, in which an *in vitro* activity relevant for the health outcome in question, guides further fractionation for a non-targeted identification of the chemicals, responsible for the activity.

protein) and have proven very valuable for diagnosing diseases or as early markers of disease onset. Thus, specific enzyme levels are used in clinical diagnostics (e.g. serum alanine aminotransferase for liver toxicity) as well as receptor levels to characterize some hormonal diseases (e.g. estrogen-dependent and non-dependent breast cancer cases). A biomarker of effect - considered as an integrated biological activity that can be measured using *in vitro* assays - has the potential to add relatively unexplored information in clinical diagnosis and/or disease onset.

In this review the state-of-the-art of the use of the integrated chemical action on endocrine receptors or other physiological targets as biomarkers of combined effects in human epidemiological studies is presented. Moreover, this review also shows the possibility to use selected *in vitro* assays as biomarkers of exposure in human samples.

1.4. An alternative way of detecting or applying biomarkers of exposure or effect in humans

A multitude of chemicals are present in human fluids and tissues, and knowledge of the linkage between these chemicals and the **integrated** adverse effect is desirable. The advantage of using receptor-based *in vitro* assays is that an integrated/combined/joint biological fingerprint of an effect is measured taking into account plausible mixture effects. Rather than measuring a single chemical or a single molecular biomarker (a protein/peptide), measurement of the total integrated effect is hypothesized to provide a more holistic and true insight into the molecular initiating events leading to the diseases, in cases where the adverse outcomes are related to a specific receptor activity. For that purpose, the top-down approaches illustrated in Fig. 1B and C can be applied. In the first approach (Fig. 1B), which is the one that has been used on human samples, an *in vitro* activity caused by a group of chemicals defined by the extraction procedure is associated with a disease outcome without identifying the specific responsible chemicals (dealt with in Section 3). In the second approach (Fig. 1C) effect-directed analysis (EDA) is used with a combination of *in vitro* tests, detailed fractionation procedures and chemical analytical methods – a tool that involves the identification of the responsible chemicals. This latter approach has been mostly applied to environmental samples and samples derived from food contact materials (FCM) (Section 2.3), but has so far not regularly been applied to human samples (Section 2.4).

We have reviewed the state-of-the art of effect-based approaches specifically applied to human samples, and briefly touch upon environmental samples and food contact materials to illustrate the applicability in the human health domain. According to our knowledge, this is the first review to present an overview of estrogen receptor- (ER), androgen receptor- (AR) and arylhydrocarbon receptor- (AhR) based *in vitro* studies performed on human tissues. We selected and included five known *in vitro* assays, covering relevant endocrine activities (*in vitro* proliferative and reporter gene assays for ER, AR and AhR, as well as *in vitro* thyroid function) to characterize the combined biological effect of chemical mixtures extracted from different human samples. Our goal was to get an impression of the maturity of the research field in order to be able to identify research gaps as well as to highlight the great potential of including receptor-based *in vitro* testing in human tissues from epidemiological studies. An inventory was compiled with an up-to-date overview of the main existing approaches in this field, further to be used as a support for the selection of more particular toxicological endpoints that can be implemented for hazard characterisation and prioritisation in future human studies.

2. Effect-directed analysis to guide identification of chemicals of emerging concern

EDA is a tool to identify chemicals of emerging concern (CECs) in a wide variety of matrices and can be used to detect biomarkers of exposure that are likely relevant from a toxicological point of view. EDA is also used to expand our knowledge on the identity of CECs present in either human, environmental or food packaging samples (Brack et al., 2016; Groh and Muncke, 2017; Jonker et al., 2015b; Tousova et al., 2017).

In the context of tracking CECs, EDA may be used in a top-down approach entailed to first characterise the biological activity associated to particular extracts and successive fractions of a human sample, then to guide the chemical identification work on this particular fraction to identify possible marker(s) of exposure responsible for this activity (Fig. 2). In EDA, both chemistry-based and biological/toxicological techniques are used in an iterative mode in order to identify the chemicals responsible for the observed activity. This approach comprises (i) biological/toxicological testing to guide the analysis towards CECs (avoiding waste of time on chemicals of no concern), (ii) fractionation to reduce the chemical complexity of the sample and (iii) identification and confirmation strategies using HRMS techniques and chemical/mass spectral databases for structural elucidation of the concerned substances. The application of *in vitro* assays inherently facilitates a prioritisation of samples, extracts or fractions to be studied for the presence of hazardous emerging chemicals by HRMS, thereby reducing the complexicity of compound identification.

The combination of these approaches may contribute to risk assessment and bring support to policy makers by providing data related both to real life human exposure and to the potential toxicity of the considered substances. Finally, results from such work will provide new candidate compounds for further evaluation and eventual inclusion in future human biomonitoring programs.

2.1. Challenges in chemical extraction and identification

To allow identification of the broad range of organic, bioactive chemicals (excluding metals) with diverse physico-chemical characteristics present in humans, it is necessary to use non-discriminating and non-destructive sample extraction and clean-up procedures. However, this ambition is facing a practical limitation considering that the

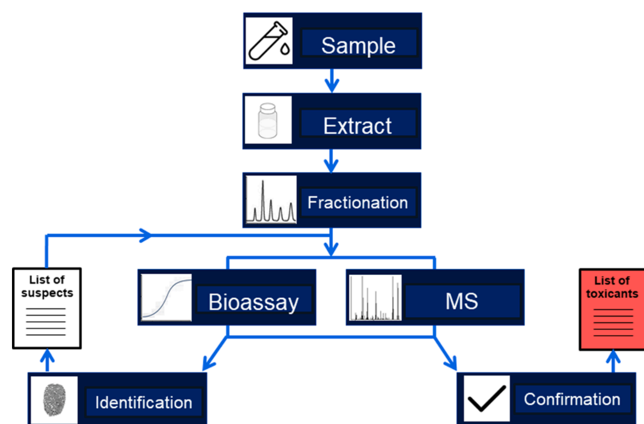

Fig. 2. Schematic representation of the building blocks of effect-directed analysis. An extract from a relevant matrix is tested in one or more selected *in vitro* assays, relevant for the research question. The active extract is fractionated into several fractions that are tested *in vitro*. The active fraction undergoes chemical analysis and tentative identification of chemicals responsible for the activity is performed. *In vitro* activity of the tentatively identified chemicals is confirmed, followed by a final identification and quantification of the newly identified CECs in the extracts.

analytical detection systems have problems in handling too complex biological samples (Simon et al., 2015). To address this complexity, high-resolution fractionation approaches resulting in fractions of time windows in the order of a few seconds have been developed based on liquid chromatography (Booij et al., 2014; Jonker et al., 2015a; Bjerregaard-Olesen et al., 2015) and gas chromatography (Pandelova et al., 2011; Pieke et al., 2013; Jonker et al., 2017). These very small fractions only contain a limited number of compounds, due to the narrow time window, thus significantly reducing the complexitiy of the fractions. To further increase the separation resolution, comprehensive two-dimensional liquid chromatography can be used for specific applications to very complex extracts (Ouyang et al., 2016). To obtain less complexity, small volume fractions could be used in combination with miniaturised *in vitro* assays. Identification is facilitated by the correlation between *in vitro* response of fractions that contain only a few chemical peaks/signals and the accurate masses obtained by mass spectrometry (Zwart et al., 2018; Zwart et al., 2020; Oberacher et al., 2020). For more details on challenges and limitations with extraction procedures and *in vitro* testing see Section 3.5.

2.2. The choice of endpoint in effect-directed analysis

Selection of the *in vitro* assays must be done carefully with respect to their ability to detect specific activities, how they associate with adverse effects in humans, and thereby their significance in human hazard identification. The choice of endpoint intrinsically drives which type of chemicals will be identified and is crucial in the context of looking for a range of CECs with various physico-chemical properties and potential biological effects that could be relevant for the human adverse outcome in question. A typical approach consists in measuring the biological activity through e.g. binding to or activation of nuclear receptors such as the estrogen receptors (ER), the androgen receptor (AR), or the aryl-hydrocarbon receptor (AhR), or the interaction with specific transport proteins e.g. transthyretin (TTR)-binding for thyroid hormone disruption or with thyroid hormone-directed cell growth.

The choice of *in vitro* assay also strongly depends on the health endpoint/effect being addressed and should be selected based on one of the molecular initiating events that are known or suspected to be associated with the adverse outcome. Thus, if the intention is to study adverse male reproductive health effects, a relevant assay to include could be an AR antagonism activity assay, whereas an ER assay would be a relevant choice for studying estrogen-related diseases such as breast cancer among other adverse female reproductive health effects.

In the next sections, we will summarise the current applications of EDA in human samples, showing the application on environmental and food packaging matrices to illustrate potential applicability in the human health domain.

2.3. Application of effect-directed analysis in environmental and food packaging samples

In biological matrices from intact organisms, only a few applications of EDA have been described and these have mainly been performed in wild-living animals. So far, the majority of EDA studies has been applied to abiotic compartments of the environment such as water and sediment (Booij et al., 2014; Houtman et al., 2006; Muz et al., 2017; Schmitt et al., 2012; Weiss et al., 2011).

The *in vitro* assays used so far have primarily been reporter gene assays for the AhR, ER, AR, binding assays for TTR, and assays for genotoxicity. To exemplify the TTR-binding assay, which measures the capacity of compounds to compete with the endogenous hormone thyroxine (T4) for binding to TTR, was used to examine thyroid hormone disruption in polar bears (Bytingsvik et al., 2013). Applying an EDA approach in polar bear plasma revealed several highly metabolised (by hydroxylation) chlorinated biphenyls as the causative contaminants (Simon et al., 2013, 2011). The classical assay based on radioactive T4,

although very reliable, was not sufficiently high throughput for effective application in EDA, and therefore a fluorescence-based modification of the assay was developed (Ren and Guo, 2012). This modified assay was miniaturized for high throughput EDA (Ouyang et al., 2017), and its sensitivity was further improved (Hamers et al., 2020).

Food contact materials (FCM) contain a large number of chemicals that can migrate to the foods and eventually enter the human body after consumption. Classical examples of these chemicals include bisphenols e.g. bisphenol A, phthalates, and/or perfluorinated chemicals, to which the majority of the human population is continuously exposed (Boberg et al., 2018; Rosenmai et al., 2016). However, employing classical approaches such as targeted analysis to characterize the chemical composition of FCMs and successively testing single compounds for biological activities is an inadequate and cumbersome strategy (Lopez-Espinosa et al., 2007; Molina-Molina et al., 2019). This procedure will neither provide information on compounds that are not explicitly known to be present in the FCMs, nor account for the total, integrated biological activities of all the compounds present in the product (i.e. the mixture effect). To address these shortcomings, an EDA strategy has been applied based on *in vitro* tests for genotoxicity (such as Ames test, p53 activation), cytotoxicity, and/or endocrine activity (such as ER, AR, AhR, PPARα/γ activity) in combination with advanced analytical chemistry to identify new CECs in FCMs (Groh and Muncke, 2017; Rosenmai et al., 2017).

2.4. Application of effect-directed analysis on human samples

EDA has to our knowledge not yet been used regularly for the identification of specific chemicals in human samples (Fig. 1C). An exception to this was the application of EDA to identify xeno-androgens in human urine samples using a human AR reporter gene assay with the purpose of using the assay read-out for doping control (studies no. 30 and 32, Table 1). Moreover, the approach was indirectly used by the selective extraction of perfluorinated chemicals (PFAS) from human blood to study the specific maternal combined PFAS mixture effect on ER activity associated with fetal growth of newborns (Bjerregaard-Olesen et al., 2019 and 2016). Challenges that may arise when doing EDA in human samples are e.g. the relatively limited sample amount available for many human matrices, the low concentrations of CECs that are to be expected in these samples and also the occurrence of metabolites (in addition to parent compounds) resulting from biotransformation that may complicate the identification of the chemicals causing the *in vitro* effects.

3. Receptor-based *in vitro* activities as biomarkers of effect

Currently, effect biomarkers are being used in order to increase the causal inference between chemical exposure (e.g. to PFAS, bisphenol A, polycyclic aromatic hydrocarbons and an effect or health outcome (e.g. neurodevelopment or reproductive diseases). With the same aim, further and complex steps have been done in many studies, in which *in vitro* assays have been applied on human samples to identify correlations between e.g. a specific nuclear receptor activation determined in a reporter gene assay and a heatlh outcome. *In vitro* activity assessment in epidemiological studies is a useful approach to directly measure a combined, specific effect of environmental chemicals included in a human biological sample, taking into account possible additive, synergistic, or antagonistic mechanisms acting in a real exposure scenario (Evans et al., 2012). The advantage of this approach is that *in vitro* assays can direct our attention to CECs as well as give us a more complete picture of the combined effect exerted by a mixture of environmental chemicals (containing both known and unknown CECs), thus approaching our knowledge to a more real-life scenario. With this approach, identification of all the specific chemicals giving rise to this specific activity (i.e. a full EDA) is usually not done (Fig. 1C). Rather the *in vitro* readout can serve either as a biomarker of effect by showing an association with a specific disease outcome, and/or as a biomarker of exposure by showing associations with demographic characteristics of a cohort population, their dietary profiles or exposure to specific chemicals (Fig. 3).

In vitro assays testing the combined biological effect of chemical fractions isolated from human samples are usually referred to as “biomarkers of combined or integrated activity'', “biomarkers of combined effect”, “biomarkers of *ex vivo* hormonal activity”, and/or “biomarkers of combined internal exposure”. These biomarkers take into account all bioactive chemicals as well as plausible interactions between these, measuring the integrated biological fingerprint, and providing a more holistic strategy to address the true cause of the disease provided that the adverse outcome is related to a specific receptor activity.

Overall, the application of *in vitro* assays in human samples such as blood, urine, amniotic fluid, milk, placentas or faeces has great potential, as it may provide insight into both known and unknown chemicals found in the human body (i.e. the specific external exposome). This knowledge is expected to support the mechanistic understanding of adverse effects through the selection of appropriate endpoints and appropriate *in vitro* assays to measure these effects (Escher et al., 2018). Additionally, the use of *in vitro* assays on human samples may provide an initial insight into whether the chemical mixture extracted from human matrices is bioactive and thereby whether this effect endpoint should be the focus of further investigations. In Table 1, an overview of these approaches is provided.

3.1. Inventory of studies on in vitro analyses of human samples

We have performed a thorough literature search and gathered an inventory of studies of receptor-based *in vitro* analyses of human samples (Table 1). The inventory includes 43 studies, in which a single or a combination of a few *in vitro* assays has been used to study associations with either exposure to POPs or with human health endpoints such as male and female reproductive diseases or breast cancer.

The majority of the studies have been performed by a Danish research group from Aarhus University (n = 16: studies, no. 9, 10, 11, 15, 16, 17, 31, 34, 36, 37, 38, 39, 40, 41, and 42) and a Spanish research group from University of Granada (n = 12: studies, no. 18, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 and 33), respectively. In seven studies (no. 1, 5, 6, 7, 8, 32, 43) scientists with relations to BioDetectionSystems in the Netherlands have been involved, whereas the remaining eight studies originate from various other research groups.

Below we will present a number of suitable *in vitro* assays that have been applied to test human samples from healthy or diseased people and discuss the obtained results.

3.2. Arylhydrocarbon receptor activation in vitro applied to human samples

3.2.1. The AhR in brief

A priority action is often focusing on halogenated organic chemicals in the screening for CECs in human samples. Indeed, this large category of compounds encompasses a broad range of exposure markers such as dioxins, polychlorinated biphenyls (PCBs), brominated flame retardants, bromo-/chlorophenols, PFAS, and chlorinated paraffins (Tarnow et al., 2019). For a subset of lipophilic halogenated persistent organic pollutants (POPs) and polycyclic aromatic hydrocarbons (PAHs), activation of the AhR is a common mechanism of action (Denison and Nagy, 2003). Consequently, *in vitro* assays based on hepatocytes expressing this particular receptor and stably transfected with a reporter gene are relevant for investigating disease associations of this large group of contaminants.

For many years the AhR was known as the ‘dioxin receptor’ as 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) was the most significant potent activator. However, endogenous compounds such as kynurenic acid, indole-3-aldehyde, indirubin and bilirubin as well as

Table 1
Overview of the use of *in vitro* assays in human samples.

No	Activity	*In vitro* assay used	Sample preparation	Human population or cohort	Observed associations to adverse health outcomes	Reference
1	*AhR activity*	AhR CALUX from BDS (rat H4IIE hepatoma cell line)	n-Hexane extraction of blood serum or follicular fluid aliquots (1–1.5 mL) and removal of acid labile matrix components	106 serum and 9 **follicular fluid** samples collected from infertile women in Belgium	AhR activity correlated well with the sum of four major **PCB congeners** (153, 138, 180, 118) chemically determined in both serum and follicular fluid	(Pauwels et al., 2000)
2		AhR CALUX from BDS (rat H4IIE hepatoma cell line)	The fat in the milk samples was extracted by addition of sodium oxalate and ethanol and subsequent extractions with diethylether and pentane. Clean-up on a silica column	16 Danish human **milk** samples	AhR activity were correlated to the TEQ levels (**PCDD/F & PCB congeners**) determined by GC/MS. Most samples gave higher TEQs with the *in vitro* test than with GC/MS	(Laier et al., 2003)
3		AhR CALUX from BDS (rat H4IIE hepatoma cell line)	2 mL blood was extracted by hexane and passaged through a silica column. DMSO was added, and the extract was diluted with minimal essential medium	101 **blood** samples from 20 to 40 year old men in Belgium	**Age** and the frequency of **fish and egg consumption** were positive determinants of serum AhR activity. Two-fold increase in AhR activity was associated with a **decreased testosterone**, a pronounced drop in semen volume but increased sperm concentration	(Dhooge et al., 2006)
4		AhR CALUX from BDS (rat H4IIE hepatoma cell line)	Total fat from 2 mL plasma was extracted in 97% hexane 3% diethylether solution.The fat extracted was then cleaned on two acid silica columns topped with sodiumsulfate (first 20% and then 30% H_2SO_4. The cleaned plasma extracts were then evaporated under nitrogen and redissolved in 8 mL DMSO	**Plasma** from 100 pregnant women selected from the Danish National Birth Cohort	Dietary patterns associated with **high fat intake** may lead to increased plasma AhR activity and in utero exposure might be related to **early infant development**	(Halldorsson et al., 2009)
5		AhR CALUX from BDS (rat H4IIE hepatoma cell line)	Blood serum was extracted with 97% hexane/ 3% diethyl ether. Passed through two acid silica columns topped H_2SO_4 to remove matrix components. Evaporated under N2 and redissolved in DMSO	**Serum** from 158 (80 cases, 78 controls) Italian women undergoing laparoscopy for endometriosis or other benign gynecologic conditions	Total AhR activity in serum was not associated with increased risk of **endometriosis**. Rather an association with PCB levels was found.	(Porpora et al., 2009)
6		AhR CALUX from BDS (rat H4IIE hepatoma cell line)	Total fat from 1 mL plasma extracted in 97% hexane/ 3% diethyl ether. Passed through two acid silica columns topped H_2SO_4 to remove matrix components. Evaporated under N_2 and redissolved in DMSO	**Blood** from 700 mothers (“Rhea” mother–child cohort study in Crete and in Barcelona)	Plasma AhR activity was negatively associated with **anogenital distance** (AGD) in male newborns. The estimated change in AGD per 10 pg TEQ/g lipid increase was –0.44 mm after adjusting for confounders	(Vafeiadi et al., 2013)
7		AhR CALUX from BDS (rat H4IIE hepatoma cell line)	Total fat from 1 mL plasma extracted in 97% hexane/ 3% diethyl ether. Passed through two acid silica columns topped H2SO4 to remove matrix components. Evaporated under N2 and redissolved in DMSO	**Blood** from 700 mothers (“Rhea” mother–child cohort study in Crete and in Barcelona)	AhR activity was higher in the children born by mothers in the upper tertile of the **“high-fat diet”** score during pregnancy, compared to the lower and middle tertiles and were positively correlated	(Papadopoulou et al., 2013)
8		AhR CALUX from BDS (rat H4IIE hepatoma cell line)	Total fat from plasma extracted in 97% hexane/ 3% diethyl ether. Passed through two acid silica columns topped H2SO4 to remove matrix components. Evaporated and redissolved in DMSO	**Breast milk** collected during the early postnatal period from mothers of 161 children born in HongKong	No association between AhR activity in breast milk and **neurocongitive function** as measured with psychological tests in 11-year-old children was found	(Hui et al., 2016)
9		AhR assay from Denison (mouse Hepa1.12cr hepatoma cell line)	POPs were extracted from 2 mL serum at Le Centre de Toxicologie, Sainte Foy, Quebec, CA, using ethanol and hexane, followed by clean-up on Florisil column	**Serum** from 338 males from Greenland (Inuit), Sweden, Poland and Ukraine	The variation of AhR serum activity may reflect different pattern of **POP** exposure, genetics and/or life style factors. No consistent correlations between AhR activities and two POP markers were found	(Long et al., 2006)
10		AhR assay from Denison (mouse Hepa1.12cr hepatoma cell line)	POPs were extracted from 2 mL serum at Le Centre de Toxicologie, Sainte Foy, Quebec, CA, using ethanol and hexane, followed by clean-up on Florisil column	**Serum** from 357 males and females from Greenland (South West, North and East Coast)	85% of the Inuit samples elicited agonistic AhR transactivity in a district dependent pattern. The AhR transactivity was **inversely correlated** to the levels of sum **POPs**, **age** and/or intake of **marine food**	(Long et al., 2007a)
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Table 1 (*continued*)

No	Activity	*In vitro* assay used	Sample preparation	Human population or cohort	Observed associations to adverse health outcomes	Reference
		AhR assay from Denison (mouse Hepa1.12cr hepatoma cell line)	POPs were extracted from 2 mL serum at Le Centre de Toxicologie, Sainte Foy, Quebec, CA, using ethanol and hexane, followed by clean-up on Florisil column	**Serum** from 70 inhabitants from 6 different Greenlandic districts, and 22 young Danish volunteers	The AhR activities of the Inuits were significantly higher than that of the Danes. AhR activity of Inuits were positively associated with **age** and 29 **plasma POPs**, whereas no correlations were found for the Danish samples	(Bonefeld-Jørgensen and Long, 2010)
12		AhR assay from Denison (mouse Hepa1.12cr hepatoma cell line)	POPs were extracted from 2 mL serum on a DSC C18 SPE column by elution with n-hexane and dichlormethane, followed by clean-up on Florisil column	**Serum** from 120 children (6–11 years) and 143 mothers living in urban or rural areas in Denmark.	PCB concentrations and AhR activities were higher in school children living in the **urban area compared with the rural area**. Strong correlation of AhR activity between **mothers and children**. AhR activity was **negatively correlated with BMI** in children and **positively with age** in mothers.	(Mørck et al, 2014)
13		AhR CALUX from XDS (mouse H1L6.1c3 hepatoma cell line stably transfected with the pGudLuc6.1 reporter)	10 g human plasma was mixed with 30 mL of acetone followed by extraction with 10 mL n-hexane. The concentrated extract (5 mL) was processed through an acid silica column in series with an activated carbon column. Dioxins were eluted and resuspended in 1.2 mL of n-hexane	**Blood** from 341 Belgian persons were collected	A significant correlation was established between the AhR activity and analytic chemical data for 17 **PCDD/F** congeners	(Van Wouwe et al., 2004)
14		AhR CALUX from XDS (mouse H1L6.1c3 hepatoma cell line stably transfected with the pGudLuc6.1 reporter)	0.5 mL whole blood was extracted with 0.5 mL *tert*-butyl methyl ether and resuspended in 200 mL hexane and dilutions of this stock extract were analysed *in vitro*	**Blood** from 10 male and female volunteers under different dietary regimens	Close to 1,000-fold higher TEQ values were found by using the *in vitro* compared to analytical chemistry, suggesting that human blood contains a relatively high level of AhR agonists apart from PCBs/dioxins /furans. A **high-vegetable diet** increased AhR activity	(Connor et al., 2008)
15	*ER activity*	MVLN assay (stably transfected human breast cancer cells expressing ERα and ERβ)	3.6 mL serum was extracted by SPE on an OASIS HLB column followed by HPLC fractionation on a Spherisorb Si60 normal phase column. The first fraction included most POPs while leaving out endogenous hormones	**Serum** from 358 males from Greenland (incl Inuits), Sweden (fishermen), Poland and Ukraine	No ER agonistic activity was found in **Inuits**, while 12–24% of the **European** samples had detectable ER activity. On the contrary, 71% of Inuit serum samples antagonised ER compared to 7–30% of the other regions	(Bonefeld-Jorgensen et al., 2006)
16		MVLN assay (stably transfected human breast cancer cells expressing ERα and ERβ)	3 mL serum was extracted by SPE on an OASIS HLB column followed by HPLC fractionation on a Spherisorb Si60 normal phase column. The F3 fraction included the PFAS. Estriol and estetrol hormones were removed by weak anion exchange	**Serum** from 397 pregnant women from Aarhus Birth Cohort, Denmark	52% of the PFAS serum extracts activated ER, and 46% enhanced the E2-induced ER activation. Positive associations were found between the **PFAS serum levels** and the ER activation	(Bjerregaard-Olesen et al., 2016)
17		MVLN assay (stably transfected human breast cancer cells expressing ERα and ERβ)	PFAAs were extracted from 3 mL human maternal serum while simultaneously removing endogenous hormones such as estrone, E2, and testosterone as described in Bjerregaard-Olesen et al.2015	**Serum** from 702 pregnant women (gestational wk11–13) enrolled in the Aarhus Birth Cohort, Denmark	An increase in PFAA-induced ER activity was associated with a 48 g decrease in **birth weight** and a 0.3 cm decrease in **birth length** in newborns.	(Bjerregaard-Olesen et al., 2019, 2016)
18		E-Screen assay: (MCF7 human breast cancer cells)	200 mg adipose tissue dissolved in hexane was eluted in a glass column with Alumine Merck 90 (70–230 mesh). The eluate was purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs, whereas the endogenous hormones were included in fraction β	**Adipose** tissue from 400 women with various diseases undergoing surgical treatment at three Granada and Almeria Hospitals, Spain	65% of the α-fractions and 76% of the β-fractions showed measurable estrogenicity. The mean estradiol equivalent unit was 750 pM EEQ/g lipid in the α-fraction and 903 pM EEQ/g lipid in the β-fraction	(Rivas et al., 2001)
19		MCF7 proliferation (human breast cancer cells)	4 mL serum was extracted with an OASIS HLB 6 cc 500 mg extraction cartridge. Slow elution by 3 mL of methanol followed by 2 mL ethyl acetate. The aqueous	**Serum** from 30 pregnant and 60 non-pregnant Danish women plus 211 samples from pregnant Faroese women	The ER activity of the serum from Danish controls exceeded the background in 23% of the cases, while the same was true for 68% of the Faroese samples	(Rasmussen et al., 2003)

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Table 1 (*continued*)

No	Activity	*In vitro* assay used	Sample preparation	Human population or cohort	Observed associations to adverse health outcomes	Reference
			phase was extracted 3 × by 1 mL n-heptane/ethyl acetate (1:1). Supernatants were reconstituted in 125 µl n-heptane /ethyl acetate (9:1)			
20		E-Screen assay: (MCF7 human breast cancer cells)	200–500 mg adipose tissue dissolved in hexane was eluted in a glass column filled with Alumine Merck 90 (70–230 mesh). The eluate was purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs whereas the endogenous hormones were included in fraction β	**Adipose** tissue from 458 women with various diseases undergoing surgical treatment in Granada and Almeria, Spain	75% of the α-fractions and 82% of the β-fractions showed measurable ER activity. The mean EEQs were 515 pM/g lipid in the α-fraction and 697 pM/g lipid in the β-fraction	(Fernández et al., 2004)
21		E-Screen assay: (MCF7 human breast cancer cells)	200 mg adipose tissue (breast tissue from cases; abdominal tissue from controls) was dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230 mesh). The eluate was further purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs, whereas the endogenous hormones were included in fraction β	**Adipose** tissues from 198 breast cancer cases and 260 age- and hospital-matched controls undergoing non-cancer-related surgery from the Granada and Almeria provinces in Spain	**Breast cancer risk was not associated** with the total ER activity in the α-fraction. However, among cases with a **body mass index** below the median (28.6 kg/m^2), OR was 2.4 for women in the highest quartile of the α-fraction activity versus those in the lowest. OR increased to 3.4 after including β-fraction activity in the model	(Ibarluzea et al., 2004)
22		E-Screen assay: (MCF7 human breast cancer cells)	1.6 g placenta homogenate dissolved in hexane was eluted in a glass column filled with Alumine Merck 90 (70–230 mesh). The eluate was purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs, whereas the endogenous hormones were included in fraction β	**Placentas** from 50 male newborn cases with congenital malformations and 114 matched controls from Granada, Spain	The total ER activity in the α-fraction was detectable in 72% and 54% of case and control **placentas**, respectively. Cases with **congenital malformations (cryptorchidism and hypospadias)** had an OR for detectable versus non-detectable ER activity of 2.8 compared to controls	(Fernandez et al., 2007)
23		E-Screen assay: (MCF7 human breast cancer cells)	1.6 g placenta homogenate dissolved in hexane was eluted in a glass column filled with Alumine Merck 90 (70–230 mesh). The eluate was further purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs, whereas the endogenous hormones were included in fraction β	**Placentas** from 489 newborns from four subcohorts in the Spanish Children's Health and Environment birth cohort study	For the tertile with the highest total effective estrogen burden of the α-fraction, higher activity was associated with **increased birth weight for boys** (β = 148.2 g), but not for girls	(Vilahur et al., 2013)
24		E-Screen assay: (MCF7 human breast cancer cells)	200 mg adipose tissue dissolved in hexane was eluted in a glass column filled with Alumine Merck 90 (70–230 mesh). No HPLC fractionation was performed	**Adipose** tissue from 386 patients (both genders) from Spain undergoing non-cancer-related surgery. 34 were type 2 diabetes patients	The total effective xenoestrogen burden was not associated with **type-2-diabetes**	(Arrebola et al., 2013)
25		E-Screen assay: (MCF7 human breast cancer cells)	1.6 g placenta homogenate was dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230 mesh). The eluate was further purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs whereas the endogenous hormones were included in fraction β	**Placentas** from 489 newborn children from four subcohorts in the Spanish Children's Health and Environment birth cohort study	No association between total ER activity in the α-fraction and the **mental scores** at age 1–2 yrs. However, assessment of **motor development** at 1–2 yrs of age, boys in the 3rd tertile of exposure scored on average 5.2 points less compared to those in the 1st tertile. The association disappeared at age 4–5 yrs	(Vilahur et al., 2014b)
26		E-Screen assay: (MCF7 human breast cancer cells)	1.6 g placenta homogenate was dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230	**Placentas** from 97 male and 95 female newborns from four subcohorts in the Spanish Children's Health and	Boys in the highest tertile of total ER activity of the α-fraction presented a decrease of 0.84% in AluYb8 **DNA methylation**	(Vilahur et al., 2014a)

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Table 1 (*continued*)

No	Activity	*In vitro* assay used	Sample preparation	Human population or cohort	Observed associations to adverse health outcomes	Reference
			mesh). The eluate was purified by HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs whereas the endogenous hormones were included in fraction β	Environment birth cohort study	compared to those in the 1st tertile, while no significant effects were found in girls	
27		E-Screen assay: (MCF7 human breast cancer cells)	1.6 g placenta homogenate was dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230 mesh). The eluate was further purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs whereas the endogenous hormones were included in fraction β	**Placentas** from 181 mother–child dyads and from 126 mother-boy dyads. The dyads were from 4 subcohorts in the Spanish Children's Health and Environment birth cohort study	No genome-wide significant associations were found between the ER activity in the α-fraction and **DNA methylation** in either study group	(Vilahur et al., 2016)
28		E-Screen assay: (MCF7 human breast cancer cells)	3 mL serum was extracted with hexane:ethyl ether (1:1). The organic phases were passed through a Bond Elut PCB cartridge. The eluate was further purified by preparative HPLC on a Spheri 5 normal phase silica column. The first HPLC fraction α contained most of the POPs whereas the endogenous hormones were included in fraction β	**Serum** from 186 breast cancer cases and 196 frequency-matched controls from a population-based multicase-control study in Spain	**Breast cancer** cases had higher total effective xenoestrogen burden of the α-fraction and the β-fraction than controls. Adjusted OR was 3.45 for women in the highest quartile of the α-fraction activity versus those in the lowest.	(Pastor-Barriuso et al., 2016)
29		E-Screen assay: (MCF7 human breast cancer cells)	200 mg adipose tissue was dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230 mesh). The eluate was purified by HPLC on a Spheri 5 normal phase silica column. The first fraction α contained most of the POPs whereas the endogenous hormones were included in fraction β	**Adipose** tissues from 55 women newly diagnosed with breast cancer from Granada, Spain. **Breast adipose** tissue was collected at the time of surgery and **abdominal adipose** tissue at the follow-up after surgery	The total effective xenoestrogen burden in both the α-fraction and the β-fraction increased during the first 6–12 mths after **breast cancer** surgery, and then decreased slightly over time	(Fernandez et al., 2017)
30	*AR activity (agonism)*	Yeast androgen assay expressing human androgen receptor and β-galactosidase	Reverse phase LC dual 96-well fraction collection set-up followed by offline LC/QTOFMS/MS and *in vitro* screening	Urine from healthy male and female volunteers	Several **androgens** were identified: Testosterone, 5α-dihydro-testosterone (DHT), androsterone, etiocholanolone, gestagen (a new undocumented designer steroid)	(Nielen et al., 2006)
31	*AR activity (agonism & antagonism)*	AR assay, transient transfected CHO cells with hAR and MMTV-LUC	POPs were extracted from 3.6 mL serum samples by SPE using Oasis HLB cartridges followed by HPLC fractionation in order to obtain the fraction containing POP mixtures but free of endogenous hormones	**Serum** from 261 adult males from Greenland, Sweden, Poland, and Ukraine	AR antagonistic activity was increased in the **Inuit** and decreased in the **Europeans**; No difference in AR agonistic activity was found	(Krüger et al., 2007)
32	*AR activity (agonism)*	AR CALUX from BDS (stably transfected human U2OS cell line)	Enzymatic deconjugation followed by liquid-liquid extraction with methyl-*tert*-butylether	**Urine** from five males and five females	Several **androgens** were identified: Testosterone, DHT, epi-testosterone, epi-DHT, androsterone, etiocholanolone	(Houtman et al., 2009)
33	*AR activity (antagonism)*	PALM reporter gene assay	1 g placenta homogenate was extracted with acetonitrile. The supernatant was filtered and fractionated by preparative HPLC on a Spherisorb Si60 normal phase column. 27 fractions were collected	**Placentas** from 29 male newborn cases with congenital malformations and 60 healthy male controls from Granada, Spain	A positive association between the total **AR antagonistic effect** of the HPLC fraction collected during 1–2 min and the risk of **malformations** (cryptorchidism and hypospadias) was found	(Arrebola et al., 2015)
34	*ER & AR*	MVLN reporter gene assay (ER), AR reporter gene assay (transient tranfections)	*ER and AR assays*: POPs were extracted from 3.6 mL serum samples by SPE using Oasis HLB cartridges followed by HPLC fractionation in order to obtain the fraction containing POP mixtures but free of endogenous hormones	**Serum** from 121 men and 119 women from Greenland	The *in vitro* activities differed between districts and genders. Associations between transactivities and **age, intake of n-3/n-6 fatty acids** and **smoker years** were observed. The ER and AR activities were correlated negatively to the POPs	(Krüger et al., 2008a)

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No	Activity	*In vitro* assay used	Sample preparation	Human population or cohort	Observed associations to adverse health outcomes	Reference
					for the combined female and male data	
35	*ER & AhR*	AhR CALUX & ER CALUX (human breast carcinoma T47D.Luc cell line stably transfected with pEREtataLuc construct)	5 mL male serum treated with 2 mL methanol was extracted 3 times with n-hexane:diethyl ether. The extracts were dissolved in 1 mL dichloromethane. For determination of ER activity, the solvent was replaced with DMSO in one-half of the crude extract; the second half of the sample was placed on a sulfuric acid-activated silica column and eluted with n-hexane: diethylether, and dissolved in DMSO	**Serum** from 150 males from residents of two areas of Eastern Slovakia, which are differently contaminated with PCBs	In human male serum samples, high levels of **PCBs** were associated with a decreased ER activity and an increased AhR activity	(Plíšková et al., 2005)
36	*ER, AR & AhR*	MVLN assay (ER), AR assay (transiently transfected) & AhR assay (Denison)	*AhR assay*: POPs were extracted at Le Centre de Toxicologie, CA as described under [9]. *ER and AR assays*: POPs were extracted from 3.6 mL serum as described under [33]	**Serum** from 262 adult males: 54 Inuit from Greenland, 69 from Poland, 81 from Sweden, and 58 from Ukraine	For Inuits negative correlations between ER and AhR activities and **sperm DNA damage** were found. In Europeans, ER, AR and AhR activities were positively correlated with sperm DNA damage	(Long et al., 2007b)
37		MVLN assay (ER), AR assay (transiently transfected) & AhR assay (Denison)	*AhR assay:* POPs were extracted at Le Centre de Toxicologie, CA as described under [9]. *ER and AR assays:* POPs were extracted from 3.6 mL serum as described under [34]	**Serum** from 319 men from Warsaw (Poland), Greenland, Kharkiv (Ukraine), and Sweden	No strong associations between receptor activities and **semen quality** was observed in any population. However, when combining data across populations, some associations between ER activity and sperm parameters were found	(Toft et al., 2007)
38		MVLN assay (ER), AR assay (transiently transfected) & AhR assay (Denison)	*AhR assay:* POPs were extracted at Le Centre de Toxicologie, CA as described under [9]. *ER and AR assays:* POPs were extracted from 3.6 mL serum as described under [34]	**Serum** from 53 Greenlandic Inuit and 247 Europeans (Sweden, Warsaw (Poland) and Kharkiv (Ukraine)	For Inuits, ER and AhR activities and **DNA fragmentation in sperm** were inversely correlated. For Europeans, a positive correlation between AR activity and DNA fragmentation was found	(Krüger et al., 2008b)
39		MVLN assay (ER), AR assay (transiently transfected) & AhR assay	*AhR–CALUX assay*: Dioxin-like POPs were extracted by SPE-Supelco using 2 mL serum as described under [12] . *ER and AR assays*: POPs were extracted from 3.6 mL serum as described under [34]	**Serum** from 31 Inuit breast cancer cases and 115 controls from Greenland	Associations between **breast cancer** risk and AR agonistic activity were found. Cases elicited a higher frequency of ER agonistic activity, but a lower AhR activity	(Bonefeld-Jorgensen et al., 2011)
40		MVLN assay (ER), AR assay (transiently transfected) & AhR assay (same as in 9 & 12)	*AhR–CALUX assay*: Dioxin-like POPs were extracted by SPE-Supelco using 2 mL serum as described under [12] *ER and AR assays*: POPs were extracted from 3.6 mL serum as described under [34]	**Serum** from 232 male and female Inuit from Greenland	The ER and AhR activities correlated negatively to the **POP** levels. POP levels were associated to **lifestyle** characteristics	(Krüger et al., 2012)
41		MVLN assay (ER), AR assay (transiently transfected) & AhR assay (same as in 9 & 12)	3 mL serum sample were subjected to SPE and liquid–liquid extraction (Hexane: EtOAc 9:1). The supernatant containing POPs was fractionated by HPLC, while the aqueous phase, containing PFAAs, was extracted with liquid–liquid extraction (tetrahydrofuran: hexane 3:2), HPLC fractionation and finally a weak anion exchange, followed by evaporation. For AhR analysis, 2 mL serum was added to a C18 SPE column and elution performed with n-hexane and dichlormethane	**Serum** from 77 breast cancer cases and 84 controls collected among Greenlandic Inuit women	A significantly **reduced breast cancer risk** was associated with higher AhR activity, but lower AR activity. No ER activities were associated with breast cancer risk	(Wielsøe et al., 2018)
42		MVLN assay (ER), AR (transiently transfected), AhR assay & T-screen	Amniotic fluid was dissolved in culture medium and directly tested *in vitro* (for ER assay: 6.25 µl/well; for AR assay: 5 µl/well; In T-screen: 14 µl/well. For AhR	**Amniotic fluids** from Danish pregnant women with birth outcome of 75 autism cases matched with 135 controls	No association between receptor activities in amniotic fluid and **autism disorders** was found. A negative correlation between	(Long et al., 2019)

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Table 1 (*continued*)

No	Activity	*In vitro* assay used	Sample preparation	Human population or cohort	Observed associations to adverse health outcomes	Reference
		(TH-dependent GH3 cell proliferation)	assay: 500 μl amniotic fluid was extracted for dioxin-like compounds by SPE and tested.		PFAS levels and autism risk was detected	
43		ER and AR CALUX from BDS (stably transfected human U2OS cell line) as well as AhR CALUX based on rat hepatoma HII4E cells	For ER and AR CALUX: Cells were exposed to 5% and 10% vol/vol plasma in cell medium. For AhR CALUX: 1 g of human plasma was extracted by hexane: diethylether, 97:3. The extract was cleaned through oxidation using an acid silica column topped with sodium sulphate	**Plasma** from 108 men with varying exposure scenarios determined by interview	Elevated AEQs were found in **smokers** and **heavy drinkers**, and in men occupationally exposed to **disinfectants** or **welding/soldering fumes**. Increased EEQs were associated with occupational exposure to **pesticides**, **disinfectants**, and **exhaust fumes**	(Brouwers et al., 2011)

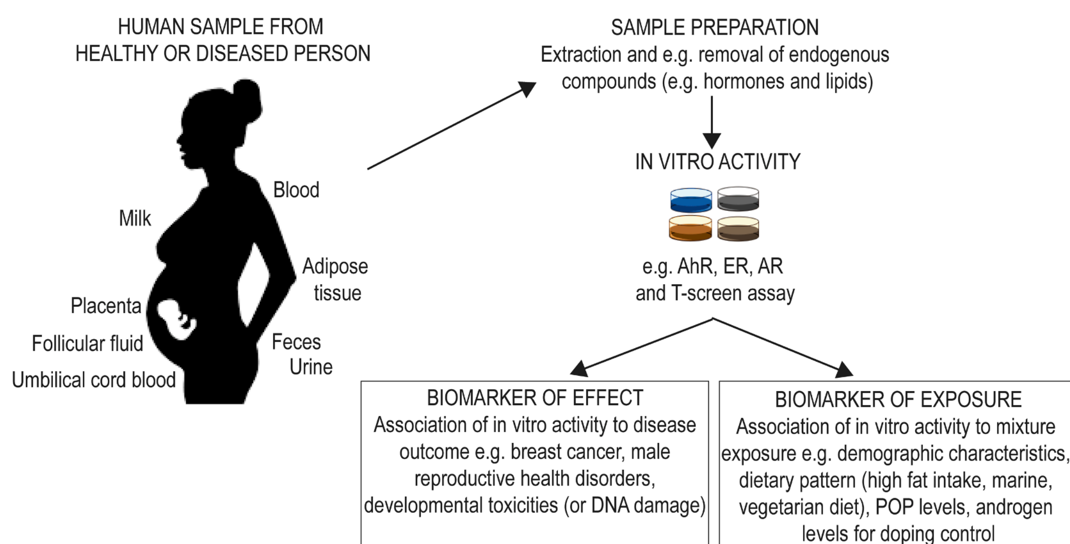

Fig. 3. Current alternative approaches for linking chemical exposures to health outcomes by using *in vitro* activities that take mixture effects of chemicals into account. Relevant human body fluids, tissues or secretions are selected from the cohort of interest and one or more *in vitro* assays analysing activation/inhibition of relevant key events for the disease in question are selected. The biological sample is typically undergoing an extraction and fractionation procedure that involves the extraction of a specific group of chemicals such as POPs or PFAS free from endogenous hormones. The measured *in vitro* acivity can be used as a biomarker of effect by association with the disease outcome, or it can be used as a biomarker of exposure by association with demographic characteristics of the cohort population, their dietary profiles or exposure to specific chemicals.

flavonoids and indole-3-carbinol have now been identified as AhR ligands as well (Denison and Nagy, 2003; Mulero-Navarro and Fernandez-Salguero, 2016). Activation of the AhR can result in an extremely diverse spectrum of biological and toxic effects that occur in a ligand-, species- and tissue-specific manner. The AhR has classically been designated an environmental sensor and has been linked to chemically-induced toxicity such as developmental toxicity, liver toxicity and carcinogenicity (Hoboken, 2012). However, the AhR has become of increasing importance as the physiologic functions of the AhR, in addition to liver metabolism and reproductive function, also include chemical and microbial defence, homeostasis of stem/progenitor cells and modulation of the immune system in barrier organs such as skin and the gastrointestinal tract (Bock, 2018). Thus, some health beneficial effects may occur at lower activity levels, whereas adverse effects may be more dominant at higher activity levels (Esser et al., 2018).

3.2.2. *Studies using AhR in vitro assays for human samples*

As many POPs are known to activate the AhR, this endpoint has been extensively used and reporter gene assays that measure AhR activation are the most thoroughly used assays on human samples. Following the dioxin crisis in 1999, where many food items such as eggs and chicken were contaminated with dioxins/furans/PCBs, several studies were conducted to assess the impact of this contamination on the dioxin body burden and for this purpose the AhR *in vitro* assay was used. AhR activity has been measured in human samples such as blood, follicular fluid, amniotic fluid or milk from various European populations. In many cases the *in vitro* responses are given as 2,3,7,8-TCDD equivalents (TEQs) and compared with the total TEQs of PCBs and dioxins/furans based on analytical quantification of these chemicals.

Methodologies for extracting POPs and testing these extracts *in vitro* for AhR activity are well-established and have proven valuable for detecting the integrated effect of all AhR agonists/antagonists (including PCBs and dioxin-like PCBs) in the sample. Comparison of these AhR activities with analytically determined PCBs and dioxins has been performed in serum, plasma or whole blood extracts (e.g. study no. 1, 13 and 14), in human milk samples (study no. 2 and 8), in follicular fluid (study no. 1) or in amniotic fluid (study no. 42).

A positive correlation of AhR activity measured in serum or blood was found with outcomes such as age (study no. 3, 11, 12), serum levels of POPs (studies no. 1, 11, 12, 35), blood levels of PCDD/F (study no. 13 and 14) as well as levels of PCDD/F and PCBs in human milk samples (study no. 2). Moreover, the AhR activities in serum of children were associated with the activities measured in their mothers, and urban citizens tended to have higher AhR activities compared to people living

in rural areas (study no. 12). The results from these studies indicate that the AhR assay is well-suited for determining the total AhR activity in various human samples including the known AhR agonists and potentially unknown AhR activating compounds.

AhR activity in blood can also be linked to diet and has been associated with intake of a high-fat diet (study no. 4 and 7) and with intake of fish and egg in the European population (study no. 3), which can be explained by the contamination pattern especially in these diets. Positive associations between AhR activity in blood and intake of a high-vegetable diet was also found (study no. 14) and in this case the AhR activity was most likely caused by natural AhR ligands (e.g. indole-3-carbinol) present in vegetables such as cabbage.

In the Greenlandic Inuit, negative correlations between AhR activity in serum and age, serum POP levels, and the biomarker of marine food intake (serum n-3 /n-6 fatty acid) was found (study no. 10). The Inuit diet includes more marine food and less terrestrial food. The n-3 fatty acids reflect marine food intake and n-6 fatty acids represent imported terrestrial food such as meat of terrestrial animals and dairy products which are known to contain many dioxin-like compounds having AhR activity. This suggests that the AhR activity mainly reflect the intake of n-6 fatty acids, which originate from a non-marine more Western-like diet such as imported food and meat products (Bonefeld-Jørgensen and Long, 2010).

High AhR activities in plasma of pregnant women have been associated with impaired early infant development (study no. 4), but no association was found with endometriosis (study no. 5). Notably, negative correlations between AhR activity and testosterone levels in adult males have been found (study no. 3) and – in line with this – also with anogenital distance in newborn boys (study no. 6). Anogenital distance is a biomarker of prenatal androgen insufficiency in rodents and humans and from a regulatory perspective classified as an adverse male reproductive health effect (Schwartz et al., 2019). 2,3,7,8-TCDD has been shown to be associated with shortened anogenital distance in humans and is known to reduce sperm quality in humans – an effect probably mediated via the AhR (EFSA, 2018). Thus, there seems to be evidence for a link between AhR activities and impaired male reproductive health.

Furthermore, an increased AhR activity in serum was associated with reduced breast cancer risk in female Greenlandic Inuit (study no. 39, 41). This is in agreement with rodent studies showing a reduced frequency of breast tumours following chronic exposure to TCDD and further supports the hypothesis of an AhR-driven anti-estrogenic action caused by increased xenobiotic metabolism and/or cross talk between AhR and ER (Tarnow et al., 2019).

Differential correlations between serum AhR activity and sperm DNA fragmentation have also been reported, with a positive correlation in the European population, and negative in Inuits (study 36 and 38, respectively). Different POPs composition, lifestyle, and/or genetic factors may influence these different correlations as AhR activation results in increased expression of enzymes involved in the metabolism of xenobiotic and endogenous compounds (Nebert et al., 2000). Substances that further enhance the dioxin-induced AhR activity may be present in the Inuit body (Long et al., 2006). Therefore, it has been speculated that the observed inverse correlation between serum AhR activity and sperm DNA damage in the Inuit might indicate a protective effect of AhR activation due to increased metabolism of compounds that stimulate sperm DNA damage causing reduced damage. However, an important role of genetic differences and/or other ethnic cofactors cannot be excluded.

The serum AhR activity is the net integrated effect of the AhR agonists and antagonists of the constituents in the mixture. While most POPs are agonists of the AhR, some PCBs and POPs can act as AhR antagonists (Hamers et al., 2006), which could actually influence the overall effect of dioxin-like compounds by decreasing the net serum AhR activity. For Inuit living far from the continent, the internal levels of dioxin-like compounds might be reduced due to selective biotransformation patterns, while non dioxin-like compounds and AhR antagonists accumulate. The composition and levels of POPs, geographical location, dietary habits and lifestyle differ between Europeans and Inuits and can have a high impact on serum AhR activity (Long and Bonefeld-Jørgensen, 2012). Consequently, the outcome of association studies between AhR activity and lifestyle factors as well as health endpoints do often differ between these populations.

3.3. Estrogen receptor activation in vitro applied to human samples

3.3.1. The ER in brief

Estrogenic activity can be measured by activation of the ERα/β in reporter gene assays or in cell proliferation assays typically based on human breast cancer cells (Soto et al., 1995). In many cases, proliferation of human breast cancer cells (i.e. MCF7 cells) have been used for testing human samples, but also ER reporter gene assays (i.e. MVLN assay and ER-CALUX®) have been extensively used. Both types of assays are based on cells that express both ERα as well as ERβ receptors, which is considered an advantage, as it allows the integrated detection on both receptors at the same time.

Estrogenic activity measurement has particularly been applied for screening of xenoestrogen disrupting chemical mixtures (Soto et al., 1997) and has been traditionally related to breast cancer risk (Darbre and Fernandez, 2013) but also to other diseases such as cardiovascular diseases (Felty, 2011), endometriosis (Caringella et al., 2011) and prostate cancer (Nelles et al., 2011).

ER agonists include primarily endogenous and synthetic steroid hormones (e.g. 17β-estradiol or ethinylestradiol, respectively), but also a wide range of environmental chemicals have been identified as activators such as bisphenols, alkylphenols, pesticides, phthalates, and parabens among others (Soto et al., 1995). However, these are generally far less potent (several orders of magnitude) than the steroidal compounds.

3.3.2. Studies on ER activity in human samples

ER activity has mainly been measured in human adipose tissue (study no. 18, 20, 21, 24, 29), in serum (study no. 15, 16, 17, 19, 28, 34, 35, 36, 37, 38, 39, 40, 41), and in placenta (study no. 22, 23, 25, 26, 27). In many cases, the output was the quantification of the estrogenic activity in tissues of European populations with various geographical locations, mainly Spain, Denmark and Greenland, and with varying environmental exposure. Among Greenlandic Inuit, correlations between ER activity and lifestyle factors such as age, intake of n-3/n-6 fatty acids and years of smoking were found (study no. 34).

A positive correlation was found between serum PFAS levels and the combined PFAS-induced estrogenic activity in serum samples from pregnant women (study no. 16). A follow-up study including a larger study population showed that the estrogenic activity of the extracted PFAS mixture from serum of Danish pregnant women was significantly associated with a decreased birth weight and length in newborns (study no. 17). Whether this was due to the estrogenic activity by itself or a secondary marker of the total PFAS exposure remains to be elucidated. However, it emphasizes the importance of analysing the combined effect of the actual PFAS serum mixture since in the same population the "one-by-one chemical approach" did not reveal any significant association to birth outcomes (Bach et al., 2016). Furthermore, it stresses the ability of some xenoestrogenic compounds, such as PFAS, to have the potential to reduce human fetal growth, which has previously been shown in several epidemiological studies (e.g. Bach et al., 2015; Fei et al., 2007).

No single chemical present in serum or adipose tissue could be associated with the biological activity measured by the MCF7 cell proliferation *in vitro* in the Spanish breast cancer case-control studies (study no. 28 and 21), with a few exceptions. However, the ER activity measured in both serum and adipose tissue samples from breast cancer patients and paired controls was positively and significantly associated with breast cancer risk (study no. 28) suggesting contributions from the mixture of measured contaminants and from unknown contaminants.

This was also the case in study no. 21, where ER activity in adipose tissue was associated with breast cancer risk, but only in women with low or normal weight, but not in those who were overweight. In patients undergoing breast cancer surgery, the ER activity in adipose tissue increased over an 18-month follow-up period, with the largest difference observed at 6–12 months post-surgery (study no. 29). In addition, breast cancer cases also elicited a higher frequency of serum ER agonistic activity compared to controls in Greenlandic Inuits (study no. 39).

Statistically significant associations were found between the adipose tissue concentrations of certain POPs and the risk of type 2 diabetes, but not with the overall ER activity measured in this tissue (study no. 24), which may indicate that estrogenicity does not play a role in the pathogenesis of diabetes.

Negative correlations between serum ER activities and increased DNA damage of sperm were found for Inuit males, whereas for European adult males (from Ukraine, Poland, and Sweden) a positive correlation with DNA damage in sperm was found (study no. 36 and 38). A potential explanation for this can be the well-known antioxidant properties of estrogens that could lead to protection against DNA damage (Adeel et al., 2017). Moreover, the populations differ with respect to POP levels and composition, dietary profiles, lifestyle, genetic factors and levels of antioxidants such as selenium. Some of these factors might explain the differential effects on sperm DNA damage in Inuit and Europeans. When combining data across the study populations ((Ukraine, Poland, Sweden, Greenland), associations between ER activity and affected sperm parameters were generally found, although no strong association between xenobiotic activity and semen quality was observed (study no. 37).

When human placenta was the matrix of choice, positive correlations between ER activity and the following adverse health effects was found: urogenital tract malformations as cryptorchidism and/or hypospadias in newborn boys (study no. 22), increased birth weight in boys, but not in girls (study no. 23), impaired motor development in 1–2 year old boys (a reversible effect that disappeared at age 4–5), but not mental scores nor any effects in girls (study no. 25).

ER activity in placenta samples has also been related to some alterations of the epigenome (DNA genome-wide methylation) as a significant association between ER activity and hypomethylation of a specific polymorphic human Alu element, which is an excellent tool for assessing population regulation and susceptibility to environmental exposure, was found in boys, but not in girls (study no. 26). Some other suggestive genes were differentially methylated in boys in relation to ER activity, but without reaching statistical significance (study no. 27).

In all the above mentioned cases, the endogenous estrogenic compounds have been removed from the human extracts before performing the *in vitro* assay (for details on methodologies in ER *in vitro* tests see Gea et al., 2020), so what is measured is the net effect of all extracted contaminants that act as either agonists or antagonists of the ER, that is the mixture effect of known and unknown ER active compounds.

3.4. Androgen receptor activation in vitro applied to human samples

3.4.1. The AR in brief

The AR is an important receptor during fetal life as the function of this receptor is vital for the normal development of the male fetus (Schwartz et al., 2019). Thus, chemicals that block this target during fetal life may adversely affect male reproductive health causing demasculinization of the newborn boys, malformation of sex organs, poor sperm quality later in life etc. (Schwartz et al., 2019).

Androgenic activity has traditionally been measured in human tissues using reporter gene assays based on either transiently transfected CHO cells, stably transfected U2OS cells or yeast cells with a read-out reflecting either agonism or antagonism of the receptor.

AR agonists are primarily endogenous or synthetic steroid hormones (i.e. testosterone, dihydrotestosterone) and only few environmental agonists have been found (Lynch et al., 2017). AR antagonists include either drugs for treatment of prostate cancer or environmental contaminants. The latter comprise a large range of chemicals including pesticides, bisphenols among other phenols, PAHs, PCBs, phthalates etc. (Vinggaard et al., 2008).

3.4.2. Studies on AR activity in human samples

In two studies being examples of true EDA on human samples, the AR assay proved useful for detecting specific anabolic androgenic steroids in human urine samples (study no. 30 and 32). Thus, this assay may have the potential to be used in doping control of athletes to detect natural and synthetic androgens. In other studies, the assay was used to discriminate between AR agonistic or antagonistic activity caused by serum POPs measured in human population segments from various geographical locations (studies no. 31, 36, 37, 38).

Analysis of serum POP extracts from Greeenlandic Inuit populations showed a positive association between AR agonism and breast cancer risk (study no. 39). Further, the breast cancer risk was significantly reduced in the group with decreasing AR activity compared to the reference group with no AR activity (study no. 41). This corroborates the view that androgens may play either primary (e.g. by stimulating the growth and division of breast cells) or secondary roles (e.g. by affecting the of androgens conversion to estradiol (E2) by aromatase activity) in breast cancer incidence (Giovannelli et al., 2018; Secreto et al., 2019; Majumder et al., 2017).

Interestingly, the AR antagonistic activity in placentas was shown to correlate with the risk of urogenital tract malformations in newborns (study no. 33). In this study, Arrebola et al. (2015) used an *in vitro*-directed fractionation methodology for the identification of (anti-) androgenic activity in five HPLC fractions of human placenta extracts, and only two of these fractions/pools were significantly associated with the risk of cryptorchidism/hypospadias. Previous studies showed that organochlorine pesticides and/or PCBs, present in the associated fractions, were significantly associated with the risk of urogenital tract abnormalities (Gaspari et al., 2011; Krysiak-Baltyn et al., 2012).

In a European population, a positive correlation between AR activity in serum and DNA damage in sperm was observed (study no. 36, 38). However, no correlation between AR activity and sperm quality measures was found in four separate European populations, but when combining data across all populations some associations between ER activity and sperm parameters were seen (study no. 37).

Overall, relatively few studies have used the combined AR activities in human epidemiological studies to assess the effect of (anti-)androgenic chemical exposure, but the present data point to an important role of the AR in female breast cancer as well in male reproductive development, which in general fits well with our current understanding. However, further research is warranted to determine whether this endpoint can be used to identify subjects particularly at risk of androgen-related diseases.

3.5. Challenges and limitations with receptor-based in vitro assays used for testing biological extracts

Sample preparation and extraction procedures are critically important, especially when investigating environmental contaminants of human biological samples. As extraction methods are rarely optimized for *in vitro* assays but rather adopted from chemical analysis, this may result in a misrepresentation of the actual biological activity. Generally, an extract of the sample is prepared in order to enrich the active compounds, which is necessay because of the often small human sample volumes/amounts as well as for removal of interfering matrix constituents. Common extraction techniques, i.e. liquid/liquid or solid phase extraction, predominantly focus on organic compounds, excluding metals. Validation of the extraction requires due attention, because routine QA/QC measures included in chemical analysis workflows like the inclusion of labeled internal standards to assess method performance, cannot be built in for effect-based testing, as the labeled standard would also provoke a response in the *in vitro* assay. In some studies,

however, chemical recovery analyses of the extract have been performed. Currently, the advancement of effect-based testing for environmental monitoring is the focus of several international collaborations and interlaboratory trials focusing on *in vitro* assays (Könemann et al., 2018; Hettwer et al., 2018, Kase et al., 2018). However, the limitations in data comparability when different laboratories use their own, non-standardized or harmonized method for the extraction of samples remains underilluminated. Nevertheless, such sample preparation and extraction techniques are routinely carried out for quantitative chemical analysis, and when due attention is paid to validation (i.e. accuracy, precision, repeatability, operational range, sensitivity, etc) these methods are very robust.

A specific challenge related to *in vitro* testing of human biological samples is the presence of endogenous hormones or molecules that can affect endocrine receptors/targets used as biomarkers in epidemiological studies. The presence of highly abundant matrix constituents such as endogenous hormones or lipids may interfere with the *in vitro* activity caused by the environmental contaminants in the human sample. For instance, if endogenous hormones are not removed, the *in vitro* readout from an estrogen assay would include the activity of endogenous hormones such as 17β-estradiol, estrone, estriol etc as well as that induced by extracted chemicals. As the integrated *in vitro* activity of contaminants in a human sample is expected to be much lower than the contributing activity of the endogenous hormones, meaning that the activity of the mixture of CECs would be at risk of being masked, it is therefore highly recommended to remove the endogenous hormones while leaving the CECs in the extract before performing the *in vitro* assay.

For most studies using receptor-based *in vitro* assays on human samples included in this review specific measures such as removing the fraction containing endogenous hormones have been applied. This has been done by isolating a fraction of a specific chemical class (e.g. lipophilic POPs or amphiphilic POPs such as the PFAS) using e.g. SPE-HPLC fractionation. In this way lipophilic compounds such as PCBs, dioxin-like compounds, organochlorine pesticides, and brominated flame retardants can be isolated in fractions that are free of endogenous hormones (e.g. Rivas et al., 2001; Fernández et al., 2004; Van Wouwe et al., 2004; Bonefeld-Jorgensen et al., 2006; Hjelmborg et al., 2006; Mørck et al, 2014). In addition, a SPE-HPLC fractionation technique to isolate a PFAS fraction from serum free of endogenous hormones has been established (Bjerregaard-Olesen et al., 2016, 2019). For both extraction methods (the lipophilic and the amphilic chemicals) recovery analyses were performed to ensure removal of endogenous hormones and to verify several compounds in the extract. These fractionation techniques allow to unravel the complexity of the extract, not only for the endogenous hormones, but also to avoid a 'masking effect' that may arise from combinations of agonists and antagonists present in the same extract (Weiss et al., 2009). When applying EDA, further fractionation is part of the workflow and the possible interference of the presence of endogenous hormones can be circumvented by excluding those fractions that account for the contribution of the endogenous hormones to the assay readout (see Section 2.1).

To ensure that the *in vitro* tests that are used on human tissues meet the minimum requirements for performance, it is advisable to use tests, for which validated test guidelines have been developed. Although currently no test guidelines are available for testing complex extracts from human tissues, validated test guidelines have been developed to test environmental water samples e.g. for genotoxicity in the Ames assay (ISO 11350, 2012). For the majority of *in vitro* test methods developed for evaluating chemical safety, no validated test guidelines are available for testing mixtures or extracts of chemicals. However, OECD has developed test guidelines for the specific ER and AR reporter gene assays, (OECD, 2016a, 2016b) that refer to the testing of pure compounds. Similarly, seventeen *in vitro* methods for testing thyroid hormone disruption are currently being assessed for a further validation study (https://ec.europa.eu/jrc/en/science-update/vitro-methods-detection-thyroid-disruptors). However, many *in vitro* methods for which no test guidelines are currently available, have been studied in interlaboratory studies (see e.g. Besselink et al., 2004) and based on the lessons learned from these studies, similar performance studies are recommended for *in vitro* assay measurements in (fractionated) human tissue extracts. Irrespective of the validation level of the *in vitro* methods, we further encourage to adhere to good *in vitro* method practices as defined by OECD (2018), which aim at reducing the uncertainties in cell-based *in vitro* method-derived predictions by applying necessary good scientific, technical and quality practices.

An additional challenge when testing biological extracts *in vitro*, is the potential interference from cytotoxic components in the complex mixture extracted from the human tissues. As data for which cytotoxicity is observed cannot be used, cytotoxicity can result in masking of the *in vitro* signal, thereby preventing detection of the relevant responses. Such challenges can be limited by thorough fractionation of the extract, thereby limiting the number of chemicals and the risk of interference in the tested fraction.

Interpretation of observed *in vitro* responses of human samples could be improved and even be standardized, if thresholds were in place that distinguish *in vitro* responses indicative for acceptable risk from responses indicative for non-acceptable risk. Such effect-based trigger values (EBTs) should correspond to *in vitro* response levels associated with increased risks of adverse outcome in the human population. The endpoint-specific EBT value could be expressed as a bioanalytical equivalent concentration, which is the concentration of a reference compound causing the same response as the threshold response. No EBT values have been derived for human samples, but the approach by Brand et al. (2013), in which ADI/TDI values were calculated into internal concentrations could be used as a good starting point.

4. Conclusions and perspectives

Forty-three published studies in which exposure and/or effect assessment was based on the use of *in vitro* assays for combined biological activity have been identified; information provided by these assays were used either for detecting CECs in human tissues, or for epidemiologically investigation on the association with human health effects.

The AhR *in vitro* tests were among the most frequently applied assays for testing of human samples. The AhR assays measure the total, integrated mixture effect of all AhR active chemical compounds (POPs as dioxins, furans, PCBs, lipophilic pesticides, flame retardants, etc.) and has been used to monitor human exposure in biological samples (e.g. blood, milk, placenta). AhR activity has been associated with male reproductive health disorders (i.e. malformations of sex organs at birth and sperm DNA damage). AhR activity has also been used for assessment of associations with dietary intake variables and was associated with intake of high fat diets, marine diets, or vegetarian diets.

Secondly, ER *in vitro* assays have been regularly applied to measure the integrated estrogenic activity in human serum, placenta or adipose tissue. Often the estrogenic activity has been shown to be associated with breast cancer, but also associations to fetal growth (birth weigth and length) and male reproductive health disorders (i.e. malformations at birth or sperm damage) have been shown.

The AR *in vitro* assays have less frequently been used on human samples, but seem to have great potential, as associations with male reproductive health disorders (i.e. malformations at birth in male newborns) and with female breast cancer have been reported.

Generally, application of EDA to human samples where *in vitro* assays are used to direct the analytical identification of CECs has been very limited; EDA approach has mostly been used to investigate environmental samples from which some lesson can be gained.

We recommend that EDA will play a more prominent role in future applications of elucidating associations between chemical mixtures exposure and adverse human health effects as a prioritization for the

identification of causative chemicals present in the human matrix. This way, the identification efforts on the HRMS data can be focused on the most relevant samples, extracts, or fractions that have biological activities defined by the selected *in vitro* assays. A huge field of novel applications waits for exploration and practical application, and we recommend researchers who design epidemiological studies to consider including *in vitro* activity measures as complementary biomarkers of effect and/or exposure in future studies. This could lead to a paradigm shift in exposure and biomarker research. The challenge is the cross-disciplinary nature of this approach that requires both chemistry and toxicological expertise together with advanced HRMS equipment as well as close collaboration with epidemiologists and clinicians.

We suggest that *in vitro* assays for especially the AR, but also for the AhR, and the ER, will be used to a much larger extent in human samples in the future. Furthermore, we expect that several *in vitro* assays for mechanisms involved in the formation or function of thyroid hormone will be applied. Some of these are used on environmental samples and are expected to be valuable for human samples as well. One putative candidate assay is the TTR binding assay which is of importance because of its capacity to transport thyroxin, but also xenobiotics, across the placenta and the blood-brain-barrier (Meerts, 2002). An overview of compounds having the potential to disrupt the thyroid hormone system and of binding to TTR of various species is available (Weiss et al., 2015). Furthermore, *in vitro* assays to identify genotoxic and mutagenic chemicals are widely used on environmental samples and FCMs to detect potential carcinogens, and could be applied to human samples as well.

In conclusion, several options exist for identifying CECs to improve assessment of human exposure to chemical mixtures. The use of *in vitro* assays combined with advanced high resolution analytical tools for suspect and non-target screening can be an advantageous way forward to direct our exposure and effect analysis in humans to mixtures of chemicals that are potentially hazardous and of concern. Thereby we should be able to identify CECs that may be amenable to public health interventions by regulatory authorities.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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