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MIXTURE ASSESSMENT OF ENDOCRINE DISRUPTING COMPOUNDS (EDC) WITH EMPHASIS ON THYROIDOGENICITY – USING CATS AS MODEL FOR INDOOR EXPOSURE

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Introduction

In 2012 a research project funded by the Swedish research council (Formas) started, abbreviated MiSSE (Mixture aSSessment of EDCs). The project consists of 4 partner Universities from Sweden and The Netherlands and will be active until 2017. The research questions in MiSSE are devoted to today's most intensely discussed subjects regarding risks of exposure to anthropogenic endocrine disrupting compounds (EDCs); 1) the most critical chemicals for exposure indoors, including not yet known or poorly studied chemicals e.g. transformation products, 2) exposure to mixtures of chemicals, 3) thyroidogenic disruption as an endocrine endpoint of growing concern for human health and the environment, and 4) improved knowledge on exposure situation during early childhood. The project is divided into 4 pillars and a central hub (Figure 1).

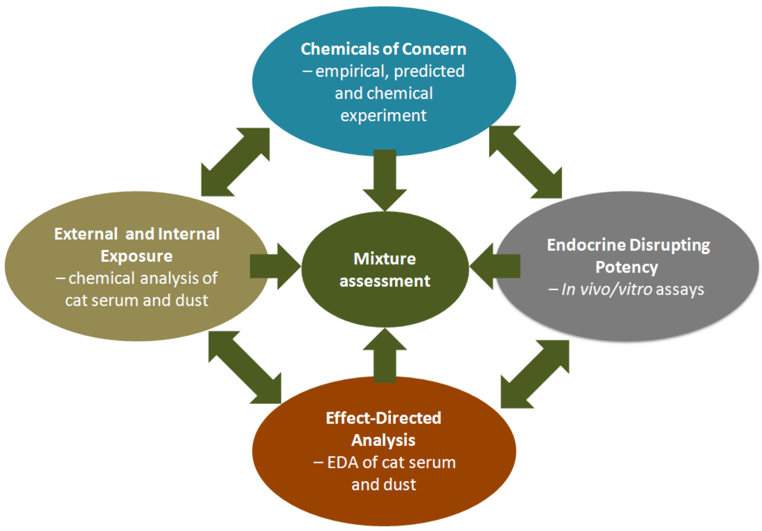

Figure 1. *Outline of the MiSSE project, represented by the pillars; the external and internal exposure (chemical analysis); chemicals of concern (in silico modelling and database compilation); endocrine disrupting potency (in vivo and in vitro bioassays); effect-directed analysis (combining chemical analysis and bioassay guidance); and the central hub mixture assessment.*

The project is aiming to assess the exposure situation to anthropogenic thyroid hormone disrupting compounds (THDCs) in homes; and accordingly the mixture effects of these compounds. A selection of key emerging THDCs are searched for by applying similarity models comparing known THDCs, and using target analysis and the effect-directed analysis (EDA) approach to indoor pet cat blood, as a model for the internal exposure to these chemicals and to dust as a route of external exposure. A battery of THDC *in vitro* screening tests and *in vivo* frog and fish models will be applied for detailed studies of individual compounds and realistic mixture signatures. As cats and toddlers have a similar behavior with their grooming and hand-to mouth activity cats are here used as sentinels for human and child exposures to indoor related chemicals and their thyroidogenic effects. Recently, a correlation was demonstrated between domestic cat's thyroid health status and levels of brominated flame retardants in their blood [1]. We will further investigate the exposure pathway via dust by correlating the levels in the two matrices dust and cat blood. Here we report the progress halfway through the project, and inform about the future perspectives regarding mixture effects and evaluation of the indoor exposure to a set of emerging compounds of concern.

More information about the project is available on the webpage at www.aces.su.se/misse/.

Samples and sampling

Seventeen families in the Stockholm/Uppsala region, Sweden, participated in the study. Samples were obtained between August, 2013 and March, 2014. The requirements of the participating families included having a healthy pet cat and at least one child living at home at an age below 10 years old. On an average the families had 2 children living at home and more than one cat. The cat's health was checked by a simple clinical examination before blood was sampled and their thyroid status was evaluated by measuring the levels of total serum T4 and thyroid stimulating hormone (TSH). All cats were clinically healthy and no one was on medication.

In total, blood was drawn from 26 unsedated pet cats in their home environment. Blood was taken from the cephalic vein in the right foreleg by a needle into plain evacuated tubes with a clotting activator. Serum was obtained by letting whole blood coagulate at ambient temperature for at least 30 min, then centrifuged for 5 min (3000 G), and the supernatant, i.e. the blood serum was collected and stored at -20°C prior to analysis. The study was performed after permission from the Swedish Board of Agriculture and Uppsala Ethical Committee on Animal Studies (No. 31-10466/12).

In parallel to the cat blood sampling, dust samples were vacuumed from three rooms of the participating families, i.e. the living room, the parent's bedroom and the child's play/bedroom. Dust were collected using a Dustream™ dust collector (Indoor Biotechnologies Ltd., Wiltshire, United Kingdom) containing a disposable filter (mesh size 40 µm) and attached on a household vacuum cleaner tube. The dust collected was so-called still standing dust, from surfaces little influenced by daily life e.g. walking and/or containing bread crumbs and soil. Typical sampling areas were book shelves, TV furniture's and around other electronics, window benches, on top of hanging and standing lamps, wall strips, but also on top of sofas and armchairs and around the beds. Sampling of human and cat hair was avoided, as well as large assembly of gravel or other influences from the outside. The sampling volume varied greatly between houses and rooms, from 40 mg up to almost 800 mg, depending on life-style and last time the room was cleaned. The participants were instructed not to clean the room for at least 3 days before sampling. The samples were stored in aluminum foil and kept at -20°C until analysis.

Details on the chemical analysis performance will be published elsewhere.

Chemicals of concern

A review of organic compounds that have been tested for their capacity to bind competitively to the thyroid hormone transport protein transthyretin (TTR) was recently published [2]. The database contains 250 individual compounds and technical mixtures, of which 144 compounds are defined as TTR-binders. Almost one third of these compounds (n=52) were even more potent than the natural hormone thyroxine (T4). In addition, the compounds tested were chemically characterized, using principal component analysis. Databases such as this one, containing compound-specific toxicological properties are important in the framework of EDA, as it could be used to assist in the identification and confirmation of causative compounds focusing on thyroid hormone disruption. In addition, this database was used to further evaluate the structure relationship to the TTR binding activity and a model was presented which can be used to predict contaminants potential capacity to bind to TTR [3]. The model was applied to a database including 485 organic dust contaminants reported from literature data and their 433 *in silico* derived metabolites. It predicted 37 parent compounds and 230 metabolites as potential TTR binders. The study presented an *in silico* approach in conjunction with bioassay to identify new THDCs and highlights the importance of metabolic activation in TTR-binding. In addition, to identify possible THDC available in the indoor environment and associated to household dust a series of abiotic transformation experiments have been conducted on one of the MiSSE model compound group, i.e. organophosphates (OP).

First results has demonstrated that the OPs undergo hydrolysis forming transformation products (unpublished). These results highlights the need to also include transformation products in the exposure analysis in the indoor environment.

External and internal exposure
Cat serum, dust and cat food were analyzed for brominated flame retardants and some phenolic metabolites (PBDEs, BB-209, DBDPE, 2,4,6-TBP, OH-PBDEs) [4]. Cat serum and cat food was in addition analyzed for organochlorines (PCBs, DDT, DDE, HCB, PCP). Correlations between cat serum, cat food and house dust in paired samples were assessed. The result demonstrated a significant correlation between cat serum concentrations and house dust levels of BDE-47 supporting the hypothesis that dust is a significant exposure route for cats. 6-OH-BDE47 was the most abundant phenolic organohalogen compounds (OHC) in cat serum. Significant correlations was found between cat serum levels and cat food for 6-OH-BDE47 and BB-209. The concentrations of BB-209 in cat serum cannot solely be explained by cat food ingestion and other sources should be investigated. DBDPE was found in high concentrations in all cat food and house dust samples, but not in any cat serum samples, suggesting DBDPE is not bioavailable.

An optimized analytical method for perfluoroalkyl acids (PFAA) analysis was demonstrated using only 50-100 µL of cat blood samples, combining online sample cleanup and LC/MS/MS analysis. The method was further slightly adopted for the analysis of polyfluoroalkyl phosphates (PFAPs). PFOS and PFDA could be determined in all cat blood samples, with an average concentration of 2300 pg/mL and 500 pg/mL respectively. Highest concentration after PFOS was for PFOA (1900 pg/mL), quantified in 96% of the samples. The concentrations of PFAPs were an order of magnitude lower than the PFAA. The 6:2 and 8:2 diPAPs were determined at highest frequency of the samples at concentrations, in 65% and 92% of the samples (1.4 – 160 pg/mL). The 6:2 monoPAP and 8:2 monoPAP concentrations could only be determined in a few samples (19-126 pg/mL). Three perfluoroalkyl phosphinic acids (PFPIA, C6/C6-, C6/C8-, C8/C8-PFPIA) could be determined in 12-28% of the cat serum. The data will be correlated to coming analyses of the house dust.

The dust and cat blood samples will be further analysed for organophosphates, parabens and phthalates.

Endocrine disrupting potency
In vivo – For the *in vivo* testing 5 chemicals have been selected as model compounds within the project, *i.e.* the tris(1,3-dichloro-2-propyl)phosphate (TDCiPP), tetrabromobisphenol-A (TBBPA), propylparaben (PrP), 6:2 fluorotelomer phosphate diester (6:2 diPAP) and Resorcinol bis(diphenyl phosphate) (PBDPP). In addition, in complementary studies or by MiSSE partners BDE-47, -99, bisphenol A bis(diphenyl phosphate) (BPA-BDPP), benzophenone 2 (BP-2) and PFOA have or will be included in the *in vivo* assays for confirmation or due to lack of data. The strategy is to first study the selected chemicals individually and then perform studies on combinations of chemicals.

Five model compounds (TDCiPP, TBBPA, PrP, 6:diPAP and PBDPP) have been tested in the zebrafish embryo toxicity tests (ZFET) in order to determine if the chemicals interfere with embryonic development. Briefly, zebrafish were exposed to chemicals from 0 to 5 days post fertilization (dpf). At 5 dpf samples were collected for qPCR analysis of genes along hypothalamus-pituitary-thyroid (HPT) axis, as preliminary analysis has shown that MISSE test compounds affect expression of these genes. The EC50, LOEC and NOEC values have been calculated and will be reported. Only 6:2 diPAP and RDP showed no developmental effects in the ZFET. In the future analysis of TRαa, TRαb an TRβgene expression levels will be performed, and a method to quantify T4 levels in the whole mount assay using a fluorescent immunoassay will be set up.

Three model compounds (TDCiPP, TBBPA and PrP) have been tested in an amphibian metamorphosis assay (AMA) with focus on detection of potential thyroid activity. The compounds have been tested using tadpoles from the amphibian *Xenopus tropicalis* in two week exposure tests. Exposure started at stage 51 (Nieuwkoop-Faber) and continued to stages 57-59, i.e. throughout the period of development where the endogenous thyroid production begins. At termination of the studies, various responses were evaluated such as body weight, body length, developmental stage, hind-limb length. Combinations of these responses were used to reveal effects on general growth or effects on developmental progress. Further, histological sections of the thyroid glands were measured regarding epithelial cell height, providing information on thyroid disruption. Methods for gene expression analysis of liver and/or tail samples are under development. Five genes: transthyretin (TTR), deiodinases (D1, D2 and D3) and thyroid receptor β (TRβ) will be analyzed which will reveal further information on the mode of action of the tested chemicals.

In vitro – Four *in vitro* assays have been used and validated to measure the thyroid hormone disrupting potency, i.e. three transport protein assays (TTR-binding, FLU-TTR and ANSA-TTR, described in [2]) and one thyroid receptor assay (TR-LUC [5]). The radioligand TTR-binding assay has been used extensively to identify THDC and was initially used for confirmation of the MiSSE model compounds [2] and the compounds suggested as THDC *in silico* [3]. A number of predicted TTR-binders and predicted non-binders parent dust contaminants were tested and accordingly, >50% of the compounds were confirmed to be predicted correctly. In addition, four new THDCs were identified as binders to TTR with IC50 values below 10µM. The FLU-TTR and ANSA-TTR were tested as alternative assays to avoid using radiolabelled thyroxin in the TTR-binding assay. FLU-TTR has been optimized and will be used further in the project.
TR-Luc has been used to confirm candidate THDC from the *in silico* modelling within MiSSE (unpublished), In general, it was concluded that the mechanism of the TR is too specific, consequently resulting in too few positive agonistic results. Hence, it was decided to continue the *in vitro* evaluation using the transport protein assays. TR-Luc was also used to evaluate the potency of the metabolites formed by hepatic activation of the suggested THDC. Only one BDE-47 metabolite and the Dicamba metabolite showed agonistic effect.

Conclusion and future perspectives
The central hub of the project is how to address complex mixtures. So far we have evaluated single compounds and performed chemical analysis to be able to reconstitute a relevant mixture of chemicals found in household dust. These artificial mixtures will be evaluated *in vitro* and *in vivo*. In addition, an EDA utilizing advanced chemical instrumentation and bioassay guidance fractionation will be performed on the household dust and cat blood in the strive to identify key-THDC. Also, EDA studies automatically include mixture aspects and disruption form metabolites and transformation products. In conclusion - cats are confirmed to be good sentinels for indoor exposures and may be applicable for a broad range of OHC exposure assessments.

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