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Developmental exposure to pyrethroids
Impact on brain and heart function

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1. Preface and acknowledgements

The presented project entitled" Developmental exposure to pyrethroids and impact on brain and heart function" was conducted in collaboration between Environmental Medicine, Institute of Public Health at SDU and DTU food in 2020-2024 and financed by the Danish Environmental Protection Agency's Pesticide Research Programme (MST-2020-67427).

The epidemiological part of the project, performed at SDU, was based on data from the Odense Child Cohort (OCC) including urinary concentrations of insecticide metabolites analyzed in a previous project supported by a grant from the Danish Environmental Protection Agency (MST-667-00164).

Results presented in this report have also been included in the following publications (status May 2024):

- Fage-Larsen, B., Andersen, H.R., Wesselhoeft, R., Larsen, P.V., Dalsager, L., Nielsen, F., Rauh, V., Bilenberg, N., 2023. Exposure to chlorpyrifos and pyrethroid insecticides and symptoms of Attention Deficit Hyperactivity Disorder (ADHD) in preschool children from the Odense Child Cohort. Environ Res, 117679. https://doi.org/10.1016/j.envres.2023.11767
- Normann, S. S., Beck, I. H., Nielsen, F., Andersen, M. S., Bilenberg, N., Jensen, T. K., & Andersen, H. R. (2024). Prenatal exposure to pyrethroids and chlorpyrifos and IQ in 7-year-old children from the Odense Child Cohort. Neurotoxicol Teratol, 103, 107352. https://doi.org/10.1016/j.ntt.2024.107352
- The pyrethroid exposure biomarker 3-phenoxybenzoic acid (3-PBA) binds to transthyretin and is associated with serum levels of thyroid hormones in pregnant women (in preparation).
- Childhood exposure to pyrethroids and chlorpyrifos and IQ-score in 7-year-old children (in preparation).
- Prenatal exposure to chlorpyrifos and pyrethroid insecticides and autism traits in preschool children from the Odense Child Cohort (in preparation).
- Effects of pyrethroid insecticides and mixtures thereof on embryotoxicity in a hIPSC-based stem cell model (in preparation).

Oral Presentation (Awarded Best Oral Presentation):

- "Predicting developmental toxicity of pyrethroid insecticides in vitro using human-induced pluripotent stem cells" at the 21st International Congress of the European Society of Toxicology In Vitro (November 2022). Abstract 276. ISBN 978-80-969474-7-8. https://www.estiv.org/congress2022/2022-estiv-congress-abstract-book/
- Presentation at a Webinar for ESTIV 2022 award winners on "New approach methodologies for evaluating cardio- and developmental toxicity" (March 2023), organized jointly by American Society for Cellular and Computational Toxicology and the European Society for Toxicology In Vitro. https://www.ascctox.org/assets/WebinarSlides/2023.03.24%20Ma.pdf

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2. Sammenfatning

Pyrethroider udgør en stor gruppe af insektmidler, som anvendes i stigende grad og den generelle befolkning udsættes især fra rester i frugt, grøntsager og kornprodukter. Desuden anvendes pyrethroider til indendørs bekæmpelse af insekter og nogle udsættes for stofferne erhvervsmæssigt. Nedbrydningsprodukter af pyrethroider kan måles i urin hos stort set alle, herunder også børn og gravide kvinder. Pyrethroider er nervegifte, som primært påvirker spændings-afhængige natrium-kanaler, der er vigtige for ledning og overførsel af elektriske impulser i nervesystemet. Da tilsvarende natrium-kanaler er væsentlige for hjertemusklens sammentrækning, mistænkes pyrethroider for også at være skadelige for hjertet men der mangler viden om dette. Desuden har den kemiske struktur af pyrethroider lighedspunkter med skjoldbruskkirtels hormoner, altså thyroideahormonerne triiodothyronin (T3) and thyroxin (T4), og menes derfor at kunne forstyrre hormonernes funktion men de mulige mekanismer er ikke fuldt ud undersøgt. Selv små ændringer i moderens niveauer af thyroideahormoner (TH) under graviditeten kan påvirke udviklingen af fosterets hjerne. Eksponering for pyrethroider i følsomme perioder i fosterliv og barndom kan derfor måske påvirke barnets udvikling og medføre øget sygdomsrisiko senere i livet. Kun få befolkningsundersøgelser har undersøgt, om der er sammenhæng mellem tidlig udsættelse for pyrethroider og nervesystemets udvikling hos børn. De fleste af disse undersøgelser omfattede under 200 børn og resultaterne er modstridende. Ingen studier har undersøgt om pyrethroider kan påvirke hjertemuskelceller fra mennesker eller om tidlig eksponering kan påvirke hjertekar-systemet hos børn.

Formålet med dette projekt var derfor at undersøge om pyrethroider binder sig til proteinet, transthyretin (TTR) som transporterer skjoldbruskkirtelhormoner i blodbanen, og om de påvirker gravide kvinders koncentration af hormonerne, og derved indirekte kan skade hjernens udvikling hos fosteret. Desuden ønskede vi at undersøge om pyrethroider påvirker deling og sammentrækning i hjertemuskelceller (kardiomyocytter) ved hjælp af en ny metode baseret på stamceller fra mennesker. Pyrethroiderne deltamethrin, α-cypermethrin, og etofenprox blev sammen med metabolitten, 3-PBA (3-phenoxybenzosyre), udvalgt til *in vitro*-testing for TTR binding og kardiotoksicitet, fordi disse tre pyrethroider var de hyppigst målte i danske fødevarer mellem 2012 og 2017 (Jensen et al., 2019). Endelig ønskede vi at undersøge om udsættelse for pyrethroider i graviditet og barndom havde betydning for børns neurologiske udvikling og deres blodtryk i barndommen. Denne epidemiologiske del af undersøgelsen er baseret på Odense Børnekohorte (OBK) hvor gravide kvinder bosat i Odense Kommune og deres børn er blevet fulgt med gentagne spørgeskemaer, indsamling af blod og urinprøver og kliniske undersøgelser herunder måling af blodtryk. Børnene er desuden blevet testet for symptomer på ADHD og autisme ved 2-4 år og ved 5 år og deres kognitive funktion (IQ) blev testet i 7-årsalderen. Nedbrydningsprodukter fra pyrethroider er tidligere blevet analyseret i urinprøver fra mødrene og langt de fleste (94%) havde målbare koncentrationer af 3-PBA, som er en fælles metabolit, der dannes og udskilles i urinen efter eksponering for langt de fleste pyrethroider. Urinkoncentrationen af 3-PBA kan derfor anvendes som mål for den samlede eksponering for pyrethroider. I det aktuelle projekt er der desuden analyseret pyrethroidmetabolitter i urinprøver fra børnene indsamlet i 5-års alderen.

Resultaterne fra projektet viste at 3-PBA bandt sig til TTR ved lave fysiologisk relevante koncentrationer, og at 3-PBA-koncentrationen i urinen hos gravide kvinder fra OBK var associeret med højere serumkoncentration af ikke-proteinbundet T3 (fT3), hvilket kan skyldes at 3-PBA har bundet sig til TTR og derved forhindret TH i at binde sig til transportproteinet. Derved kan transporten af TH til fosteret under et meget sårbart udviklingsvindue være forstyrret. Vi fandt dog ikke nogen statistisk signifikante sammenhænge mellem mødrenes 3-PBA-koncentration under graviditeten og øget risiko for autisme symptomer i 2-4-årsalderen eller ADHD-symptomer i 5-årsalderen eller nedsat kognitive evner (IQ) i 7-års alderen. Vi har tidligere fundet at 3-PBA i mødrenes urin var relateret til højere ADHD-score blandt børnene i 2-4-årsalderen (Dalsager et al., 2019) men denne sammenhæng var altså ikke længere registrerbar i 5-års alderen. Vi fandt heller ingen sammenhæng mellem børnenes egen pyrethroideksponering ved 5 år og ADHD-symptomer ved 5 år eller IQ-score ved 7 år.

Med hensyn til kardiotoksicitet blev alle tre testede pyrethroider, men ikke 3-PBA, fundet at nedsætte differentieringen af kardiomyocytter. Effekten var koncentrationsafhængig og signifikant allerede i det mikromolære område. Denne kardiotoksiske virkninger blev ikke afspejlet i højere blodtryk relateret til tidlig pyrethroideksponering hos børnene i OBK.

Resultaterne fra projektet understøtter, at pyrethroider har TH-forstyrrende og kardiotoksiske egenskaber. At der ikke kunne påvises signifikante sammenhænge mellem tidlig pyrethroideksponering og børnenes neurologiske udvikling eller blodtryk i OBK, kan skyldes en lav men udbredt pyrethroideksponering i denne kohorte. Der kunne således måles 3-PBA i næsten alle urinprøver men variationen i koncentrationen var lav, hvilket gør det svært at påvise en eksponeringsrelateret effekt. Ydermere var 3-PBA-koncentrationerne lave sammenlignet med de fleste andre studier i EU, der har anvendt human biomonitorering til at fastlægge eksponeringen. Koncentrationerne var også betydeligt lavere end 3-PBA-koncentrationer rapporteret fra undersøgelser i Asien og USA med urinprøver indsamlet i samme periode. Især 3-PBA-koncentrationer blandt børnene i dette projekt var lave sammenlignet med andre undersøgelser og de var også lavere end hos mødrene, hvilket kunne indikere et skift i retning af højere indtag af økologisk mad i familierne, efter at børnene blev født i denne ret veluddannede kohorte.

Resultaterne fra dette projekt har bidraget med ny viden om toksiske mekanismer for pyrethroider, der kan have betydning for langtidseffekter i sårbare befolkningsgrupper, som bør inddrages i myndighedernes regulering af disse stoffer. Den observerede sammenhæng mellem 3-PBA og fT3 hos mødrene i OBK er bekymrende og pyrethroiders TH-forstyrrende egenskaber bør undersøges nærmere, inklusiv de mulige sundhedsmæssige konsekvenser. Det vil i den sammenhæng være relevant at inddrage data fra kohorter med forskellige eksponeringsniveauer for bedre at kunne undersøge dosis-responsrelationer og fastlægge sikre eksponeringsniveauer. Da brugen af pyrethroider har været stigende, er det desuden meget relevant at følge eksponeringsniveauet af pyrethroider i befolkningen.

3. Summary

Pyrethroids compose a large group of insecticides increasingly used in agriculture, and the general population is mainly exposed from residues in foods, especially fruit, vegetables, and cereals. Metabolites of pyrethroids are widely detectable in urine samples from the general population, including pregnant women and children. Pyrethroids are neurotoxicants acting primarily by interfering with voltage-gated sodium channels that are vital for conduction and neurotransmission in the nervous system. Since voltage-gated sodium channels are also essential for cardiac muscle contraction they are suspected also to be cardiotoxic, but the knowledge is currently limited. Besides, pyrethroids have structural resemblance to thyroid hormones (THs), i.e., triiodothyronine (T3) and thyroxine (T4), and have been suggested to interfere with thyroid hormones but the exact mechanisms have not been fully explored. Even subtle changes in maternal thyroid hormones can affect fetal brain development. Thus, exposure to pyrethroids during vulnerable time windows in pregnancy and childhood may have long-term impact on child neurodevelopment and cardiovascular health. Relative few previous human studies have investigated association between pyrethroid exposure in utero and early childhood and neurodevelopment. Most of these studies have included less than 200 children and the results were conflicting. No previous studies have investigated if pyrethroids can affect human cardiomyocytes or the potential impact of early pyrethroid exposure on the cardiovascular system in children.

Thus, the purpose of this project was to investigate if pyrethroids bind to transthyretin (TTR), the specific transporter protein for thyroid hormones in blood and cerebrospinal fluid, and whether maternal thyroid function in pregnancy was related to pyrethroid exposure. Further, we aimed to investigate and if pyrethroids affect cardiomyocyte differentiation *in vitro* using a newly developed method based on human-induced pluripotent stem cells (hiPSC). The pyrethroids deltamethrin, α-cypermethrin, and etofenprox were selected for testing of TTR binding and cardiotoxicity *in vitro* as they were the most detected pyrethroids in Danish food products between 2012 to 2017 (Jensen et al., 2019). In addition, the metabolite 3-PBA (3-phenoxybenzoic acid) used as exposure biomarker for the total pyrethroid exposure, was included. Finally, we aimed to investigate if prenatal and/or childhood pyrethroid exposure was associated with neurodevelopment and blood pressure during childhood. The epidemiological part pf the project is based on the Odense Child Cohort (OCC), which is a large ongoing prospective Danish birth cohort, in which pregnant women residing in Odense Municipality between 2010 and 2012 were recruited. The mothers and their children have been followed with repeated questionnaires, collection of blood and urine samples, and clinical examinations including measurement of blood pressure. The children have also been tested for symptoms of ADHD and autism at 2-4 years and at 5 years and their cognitive function (IQ) was tested at 7 years of age. Metabolites of pyrethroids have previously been analyzed in urine samples from the mothers and the majority (94%) had measurable concentrations of 3-PBA, which is a common metabolite formed and excreted in the urine following exposure to most pyrethroids. The urinary concentration of 3-PBA can therefore be used as a measure of total exposure to pyrethroids. As part of the current project, pyrethroid metabolites were analyzed in urine samples from the children collected at the age of 5.

The results from the project showed that the pyrethroid metabolite 3-PBA, was able to bind to TTR at low physiological relevant concentrations, and urinary 3-PBA concentrations were associated with higher non-protein bound T3 (fT3) among pregnant women in the OCC, which may be due to binding of 3-PBA to TTR causing displacement of TH from TTR. Displacement of TH from TTR in early pregnancy may disturb the transplacental transport of TH to the fetus

during a very vulnerable window of development. However, we did not find any statistically significant associations between maternal urinary 3-PBA concentrations and increased risk of autism symptoms at age 2-4 years or ADHD symptoms at age 5 years or with reduced cognitive function (IQ) at age 7 years. Thus, our previous finding from the OCC of a significant association between prenatal pyrethroid exposure and higher ADHD scores at 2-4 years-of-age (Dalsager et al., 2019) was no longer apparent at age 5 years. Further, low pyrethroid exposure in childhood at age 5 years was not significantly associated with higher risk of ADHD symptoms at age 5 years or lower IQ-scores at age 7 years.

Regarding cardiotoxicity, all three tested pyrethroids (deltamethrin, α-cypermethrin, and etofenprox), but not 3-PBA, were found to impair cardiomyocyte differentiation in a concentration-dependent manner, with significant effects in the order of low micromolar levels. These cardiotoxic effects were not mirrored in higher blood pressure related to early life pyrethroid exposure in the OCC.

The results from this project support that pyrethroids have TH disruptive and cardiotoxic properties. The lack of significant associations between prenatal or childhood pyrethroid exposure and adverse effects on neurodevelopment or blood pressure in the OCC, is likely attributable to a low but widespread exposure to pyrethroids in this cohort. Thus, the common pyrethroid metabolite, 3-PBA, was detectable in almost all urine samples but the variation in the concentrations was low. Such a narrow exposure gradient hampers the possibility to detect an exposure related effect. Further, the 3-PBA concentrations were low compared to most other biomonitoring studies performed in the EU using human biomonitoring to assess exposure. The concentrations were also considerably lower than 3-PBA concentrations reported from studies in Asia and the US with urine samples collected within the same years. Especially 3-PBA concentrations among the children in this project were low compared other studies and they were also lower than the maternal concentrations which may indicate a shift towards higher intake of organic food in the families after the children were born within this rather well-educated cohort.

The results from this project have contributed with new knowledge about toxic mechanisms of pyrethroids, of potential relevance for long-term health effects in vulnerable population groups, which should be included in the regulation of these substances by the authorities. The observed association between 3-PBA and fT3 in the mothers in OCC is of concern and the TH-disrupting properties of pyrethroids deserves to be investigated further, including the potential health impacts. In this context, it will be relevant to include data from cohorts with different exposure levels to better investigate dose-response relationships and determine safe exposure levels. As the use of pyrethroids has been increasing, it is also very relevant to follow the exposure level of pyrethroids in the population.

4. Introduction

4.1 Background

4.1.1 Pyrethroid exposure

Pyrethroids compose a large group of insecticides used worldwide in agriculture, indoor environments, and for vector control (Lehmler et al., 2022; van den Berg et al., 2021). Pyrethroids use has been increasing during the last decades, as they replace other insecticides, e.g., organophosphates that are bound by regulatory restrictions due to acute toxicity or concern for carcinogenicity and developmental neurotoxicity (EFSA, 2019). In 2015, pyrethroids represented approximately 38% of the world insecticide market (Li et al., 2017). In geographical areas where indoor use of pyrethroids is limited, the general population is mainly exposed from residues in food (Baudry et al., 2019; Schettgen et al., 2002). After ingestion, pyrethroids are extensively absorbed from the gastrointestinal tract, rapidly metabolized and excreted, primarily in the urine, within a few days. However, due to high lipophilicity, a minor fraction is absorbed through the lymphatic pathway thereby by-passing hepatic first pass metabolism (Mallick et al., 2020) and at continuous low exposure, pyrethroids achieve steady-state levels in internal tissues (Cote et al., 2014; EFSA, 2011). They are capable to cross the placenta and both the parent compounds and some metabolites have been detected in meconium (Berton et al., 2014) and umbilical cord blood (Neta et al., 2010; Silver et al., 2016).

Urinary concentrations of pyrethroid metabolites are used as biomarkers for the internal exposure level integrating all exposure routes. The most commonly used urinary biomarkers are the generic pyrethroid metabolite, 3-PBA (3-phenoxybenzoic acid), representing exposure to most pyrethroids; cis- and trans-DCCA (3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid) representing exposure to the cis- and trans-isomers of permethrin, cypermethrin, and cyfluthrin; 4-F-3PBA (4-fluoro-3-phenoxybenzoic acid), a specific metabolite of cyfluthrin; and cis-DBCA (cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid), a specific metabolite of deltamethrin. The urinary biomarkers reflect recent exposure and high within-individual variability in metabolite concentrations has been reported (Li et al., 2019; Morgan et al., 2016). However, in populations exposed mainly from residues in the diet, the total pyrethroid exposure can be assumed to be rather continuous. Thus, the urinary 3-PBA concentration in spot urine samples is considered a valid biomarker for the aggregated exposure to dietary mixtures of pyrethroids among individuals from the general population, although it may not capture peak exposures from e.g., indoor use. Accordingly, 3-PBA was detected with similar detection rates and geometric mean concentrations across pregnancy among women who provided repeated samples (Barkoski et al., 2018; Watkins et al., 2016). Urine concentrations of the more specific metabolites (trans- and cis-DCCA, cis-DBCA and 3-F-PBA) are dependent on recent exposure to specific pyrethroids and will therefore, most often, have a lower detection rate and a higher intra-individual variation.

The exposure level, estimated by urinary 3-PBA concentrations, has increased during the last decades in some regions (Lehmler et al., 2022), e.g., in the US (CDC, 2015), Canada (CHMS, 2013), and Sweden (Noren et al., 2020). Thus, 3-PBA is now widely detectable in urine from the general population including pregnant women in Denmark (Dalsager et al., 2019) and elsewhere (Andersen et al., 2022b; Dereumeaux et al., 2018; Lee et al., 2022), although at lower concentrations in individuals who predominantly eat organic food (Baudry et al., 2019). Children are generally higher exposed than adults because they eat more food per kg body weight (Andersen et al., 2022b; Bravo et al., 2020). Accordingly, the rise in exposure level was reported to be highest among children (Jain, 2016).

4.1.2 Developmental neurotoxicity

Pyrethroids have low acute mammalian toxicity, but concern for long-term health effects at population exposure level has increased (Demeneix et al., 2020). Pyrethroids target the nervous system of insects primarily by preventing closure of voltage-gated sodium channels in axonal membranes (Soderlund, 2020). Voltage-gated sodium channels are integral membrane proteins. Pyrethroids modify the function by binding to unique receptors on the pore-forming subunits of the sodium channels preventing its transition from an activated (ion-conducting) to an inactivated (non-conducting) state. As a result, the membranes of electrically excitable cells become persistently depolarized, and the insect is paralyzed and killed. Since the structure and function of these channels are highly conserved between insects and mammals, pyrethroids also alter the function of mammalian sodium channels and exhibit neurotoxic properties in non-target organisms including humans (Abreu-Villaca and Levin, 2017; Soderlund, 2020). However, higher metabolic capacity and lower sensitivity of mammalian voltage-dependent sodium channels towards pyrethroids offer some protection against their toxicity (Soderlund, 2020).

The developing brain is particularly vulnerable to neurotoxicants, and exposure during fetal and early life may have long-term impact on neurodevelopment (Grandjean and Landrigan, 2006; Grandjean and Landrigan, 2014; Rice and Barone, 2000). Long-lasting alterations in brain function related to prenatal or early postnatal pyrethroid exposure have been demonstrated in animal models including a range of neurochemical and neurodevelopmental alterations, e.g., hyperactivity and deficits in learning and memory (Abreu-Villaca and Levin, 2017; Pitzer et al., 2021). Some of these studies were performed at high sublethal doses but effects were also seen at low doses more relevant for human exposure levels. Oral exposure of female mice to deltamethrin during gestation at doses (0.3, 1, or 3 mg/kg) that span the current NOAEL of 1 mg/kg, caused male offspring to exhibit hyperactivity and impulsive-like behaviors that were interpreted as features of attention deficit hyperactivity disorder (ADHD) (Richardson et al., 2015a). A subsequent study from the same group and similar exposure conditions, found significant downregulation of the expression of genes for multiple sodium channel subunits and brain-derived neurotrophic factor (BDNF) in offspring at 10–11 months of age (Magby and Richardson, 2017). Also, low doses (1.2 mg/kg) of either cypermethrin or deltamethrin administered intragastric to pregnant female mice from gestation day 10.5 to 16.5 caused a reduction in neuronal proliferation, cell maturation and differentiation, and increased apoptosis in the cerebral cortex of new-born offspring (Guo et al., 2018). Further, intranasal administration of low doses (5 and 20 mg/kg) of cypermethrin to female mice from gestation to postnatal day 15 caused disturbed motor development and maladaptive behaviors in response to highly challenging tasks and abnormal sociability in the offspring (Laugeray et al., 2017).

Some studies have investigated associations between pyrethroid exposure during pregnancy and neurodevelopment in human populations. In a previous study from the OCC, maternal urinary concentration of 3-PBA in pregnancy was associated with higher ADHD scores among the children at age 2-4 years (Dalsager et al., 2019). This finding was in accordance with other birth cohort studies reporting associations between maternal pyrethroid metabolites and delayed mental development at 2 years of age (Watkins et al., 2016) and behavioral problems among preschool and school age children (An et al., 2022; Furlong et al., 2017; Lee et al., 2022; Viel et al., 2017) or reduced IQ at school age (Tanner et al., 2020). Higher maternal exposure levels in agricultural settings or from indoor use for malaria control have also been associated with cognitive deficits (Eskenazi et al., 2018; Gunier et al., 2017; Xue et al., 2013). In contrast, maternal pyrethroid metabolites were not associated with cognitive deficits at school age in a French birth cohort (Viel et al., 2015) or developmental scores at 18 months of age in a Japanese cohort (Hisada et al., 2017). In the OCC, we did not find associations with delayed language development at age 2-3 years (Andersen et al., 2021a), but cognitive deficits may manifest later in childhood because of cascading developmental processes and better opportunity for examination of complex cognitive functions.

Since growth and functional development of the human brain continues during childhood, it is assumed that the postnatal period is also vulnerable to neurotoxic exposures (Grandjean and Landrigan, 2006). Accordingly, childhood pyrethroid exposure (child urinary concentrations of pyrethroid metabolites) has been associated with impaired cognitive functions at age 3-6 years (Wang et al., 2016) and 6-9 years (van Wendel de Joode et al., 2016; Viel et al., 2015) and increased risk of behavioral problems (Lee et al., 2020; Oulhote and Bouchard, 2013; Viel et al., 2017) including ADHD (Wagner-Schuman et al., 2015). However, these studies were all cross-sectional and therefore reverse causality, i.e., that delayed neurodevelopment cause higher exposure, cannot be excluded although it seems unlikely. However, a recent study found child urinary 3-PBA concentrations at age 2 to 6 years to be associated with higher ADHD scores at age 6 and 8 (Lee et al., 2022).

Overall, most previous epidemiological studies found associations between pyrethroid exposure during vulnerable periods in pregnancy or childhood and impaired neurodevelopment (Andersen et al., 2022a). However, the studies used a variety of different methods to assess neurodevelopment, few studies investigated cognitive function, most were performed in children below 4 years of age, and approximately half of the studies were of modest sample size of less than 300 children (Andersen et al., 2022a).

4.1.3 Thyroid hormone disruption

Pyrethroids and some metabolites, including 3-PBA, have structural resemblance to thyroid hormones (THs), i.e., T3 and T4 (Figure 1) and have been suggested to be thyroid hormone (TH) disruptors based on experimental studies (Du et al., 2010; Ghisari et al., 2015; Leemans et al., 2019; Zhang et al., 2020).

Maintenance of normal thyroid function is important for numerous physiological processes but especially pregnancy is a vulnerable period, and both the mother and fetus are sensitive to even minor disturbances (Boas et al., 2012; Jansen et al., 2019). Since the human fetus is unable to synthesize TH during the first months of pregnancy, placental transfer of maternal TH plays a pivotal role in early fetal development and it is well-known that even subtle changes in maternal TH function in early pregnancy can affect fetal brain development (Jansen et al., 2019; Moog et al., 2017; Mughal et al., 2018). However, maternal thyroid homeostasis is important for optimal fetal development during the whole pregnancy (Boas et al., 2012; Jansen et al., 2019).

The control of TH homeostasis is complex and can be disturbed by environmental chemicals through a variety of different mechanism (Boas et al., 2012; Noyes et al., 2019). Among these, competitive binding to the transport protein transthyretin (TTR) has been identified as an important TH disruptive mechanism for several environmental chemicals such as hydroxylated PCBs, brominated flame retardants and perfluorinated chemicals in experimental studies (Chang et al., 2008; Hallgren et al., 2001; Ouyang et al., 2017; Rosenmai et al., 2021; Weiss et al., 2009). Although TTR binds only a minor proportion of TH in humans, TTR has been proposed to be of special importance for transferring of TH over the blood–brain barrier as well as over placenta to the fetal compartment (Boas et al., 2012; Noyes et al., 2019; Richardson et al., 2015b). Further, TTR is an important carrier of T4 in cerebrospinal fluid and is therefore essential for the function of TH in brain development (Moog et al., 2017). So far interaction with TTR has only been investigated for one pyrethroid, permethrin, in a model using embryonic zebrafish. In that study, permethrin was found to interact with the binding pocket at TTR and to increase the gene expression of thyroid-stimulating hormone (TSH), deiodinases, TH receptors and the concentrations of T4 and T3 (Tu et al., 2016). Thus, it is highly relevant to investigate whether TTR binding is a more general mechanism of action for pyrethroids.

Few epidemiological studies have investigated potential disturbance of TH function related to pyrethroid exposure among pregnant women. In a Japanese birth-cohort study, no associations were seen between urinary 3-PBA concentrations and TH concentrations in serum among 230 pregnant women in gestational week (GW) 10-12 (Zhang et al., 2013) or serum from their new-borns (Zhang et al., 2014). However, other studies found maternal 3-PBA to be associated with lower concentrations of free T3 (fT3) in third trimester (Hu et al., 2019), lower thyroid-stimulating hormone (TSH) in serum samples collected before GW 33 (Corrales Vargas et al., 2022), or increased concentrations of TSH in new-borns (Chevrier et al., 2019). The lack of consistency across the studies could be due to the variation in study design, including differences in timing of sample collection during pregnancy, hormones measured, population demographics, and level of pyrethroid exposure. Thus, more studies are needed to investigate if pyrethroid exposure can disturb TH function in pregnancy, eventually by binding to TTR and disturbance of the transport of THs from the maternal circulation to the fetal brain, and in this way disturb fetal brain development.

4.1.4 Cardiotoxicity

While sodium channels are paramount to nerve conduction and neurotransmission, they are also vital for skeletal and cardiac muscle contraction (Catterall et al., 2020). Especially muscle cells in the heart, cardiomyocytes, are rich in sodium channels but, compared to nerve cells, little is known about the effect of pyrethroids on the heart. One study using isolated cardiomyocytes from guinea pigs and rats and perfused rat hearts found that tefluthrin and fenpropathrin and cypermethrin prolonged action potentials and evoked after-depolarizations and increased the variability of contractile amplitude between one heartbeat and the next (Spencer et al., 2001). Similar arrhythmogenic effects were seen for deltamethrin in perfused hearts and isolated cardiomyocytes from the crucian carp (Haverinen and Vornanen, 2016). *In vivo*, rats exposed to permethrin (1/50 LD50) from 6th to 21st day after birth exhibited decreased heart cell membrane fluidity, cardiac hypotrophy, increased intracellular calcium, and other indicators of cardiotoxicity in adulthood (Vadhana et al., 2013; Vadhana et al., 2011). However, no previous studies have investigated potential effects of pyrethroids on human cardiomyocytes. A 3D model of human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes has been developed (Lauschke et al., 2020) and this experimental model may bring us a step closer to unravel the effects and the molecular initiating events for cardiotoxicity caused by pyrethroids.

Only two epidemiological studies have so far addressed potential cardiovascular effects of pyrethroids in humans. A study, based on 2116 adults from the US National Health and Nutrition Examination Survey (NHANES), found higher risk of all-cause and cardiovascular disease mortality among participants with the highest tertile compared with those with the lowest tertile of 3-PBA analyzed in urine samples collected between 1999 and 2002 (Bao et al., 2020). The exposure data were linked to mortality data in December 2015 with a median follow-up time of 14.4 years. After adjustment for age, sex, race/ethnicity, socioeconomic status, dietary and lifestyle factors, BMI, and urinary creatinine levels, the hazard ratio for cardiovascular disease mortality was 3.00 (95% CI, 1.02-8.80). This finding was confirmed in an updated study including data from both the 1999-2002 and 2007-2012 HNANES surveys in which participants in the highest tertile of urinary 3-PBA had higher odds of cardiovascular disease (OR: 1.58; 95% CI: 1.12, 2.23) and coronary heart disease (OR, 1.75; 95% CI: 1.17, 2.61) compared to those in the lowest tertile (Xue et al., 2021). The findings was also supported by a previous case-control study of 72 patients with coronary heart disease and 136 healthy individuals in China, in which pyrethroid exposure was associated with higher odds of coronary heart disease when comparing the highest with the lowest tertile of urinary pyrethroid metabolite concentrations (OR for total metabolites: 4.55 (95% CI, 1.80-11.54)(Han et al., 2017). No studies have investigated the potential impact of early pyrethroid exposure on the cardiovascular system in children.

4.2 Aims of the project

We hypothesized that exposure to pyrethroids during vulnerable time windows in pregnancy and childhood may have long-term impact on child neurodevelopment and cardiovascular health. Thus, the purpose of this project was to investigate if prenatal and/or childhood pyrethroid exposure was associated with neurodevelopment and blood pressure during childhood in a large prospective cohort of approximately 1000 mother-child pairs and whether maternal thyroid function in pregnancy was related to pyrethroid exposure. Further, we aimed to investigate if pyrethroids bind to transthyretin (TTR), the specific transporter protein for thyroid hormones in blood and cerebrospinal fluid, and if pyrethroids affect cardiomyocyte contractions *in vitro* as potentially relevant mechanisms.

The epidemiological part of the project is based on the Odense Child Cohort (OCC), which is a large ongoing prospective Danish birth cohort. In this cohort we had previously analyzed pyrethroid metabolites in 1200 maternal urine samples collected in pregnancy. The generic pyrethroid metabolite, 3-PBA, was detectable in almost all (94%) maternal samples and the concentration was associated with higher ADHD scores among the children at age 2-4 years (Dalsager et al., 2019). We have also previously analyzed pyrethroid metabolites in 460 urine samples collected from the children at age 5 years, and as part of this study we wanted to analyze additional 400 urine samples from children for whom we had data on neurodevelopment.

The specific aims of this project were to investigate if:

- Neurodevelopment in children, ADHD and autism scores at 2.5 and/or 5 years of age and an IQ test at age 7 years, was associated with maternal and childhood urinary pyrethroid metabolite concentrations.
- Maternal pyrethroid exposure was associated with altered thyroid hormone concentrations during pregnancy.
- Alterations in maternal thyroid function could explain (mediate) potential associations between prenatal pyrethroid exposure and adverse child neurodevelopment.
- Pyrethroids* bind to transthyretin (TTR) using an established ANSA-TTR displacements *in vitro* assay.
- Pyrethroids* affect cardiomyocyte beating using a recently developed *in vitro* model based on human-induced pluripotent stem cells (hiPSC) that are differentiated into cardiomyocytes.
- Higher blood pressure measured repeatedly during childhood at 18 months, 3, 5 and 7 years of age were related to prenatal pyrethroid exposure, and/or for the two oldest age groups, also to childhood exposure.

*The pyrethroids deltamethrin, α-cypermethrin, and etofenprox were selected for testing in the *in vitro* assays as they were the most detected pyrethroids in Danish food products between 2012 to 2017 (Jensen et al., 2019). In addition, the metabolite 3-PBA, used as exposure biomarker for the total pyrethroid exposure, was included.

The organophosphate insecticides chlorpyrifos and chlorpyrifos-methyl were banned for use in the EU in 2020 partly because of concern for developmental neurotoxicity (EFSA, 2019). Since a specific urinary biomarker (TCPy) for these two compounds were analyzed along with the pyrethroid metabolites in the OCC samples, we decided to present these exposure data and to include TCPy in the data-analyses of neurodevelopmental outcomes in this report. We thought these results were relevant for comparison with 3-PBA, since pyrethroids might partly replace chlorpyrifos/chlorpyrifos-methyl.

5. Materials and methods

5.1 Experimental studies

5.1.1 Chemicals and reagents

Deltamethrin (CAS: 52918-63-5, purity: 98.6%), Etofenprox (CAS: 80844-07-1, purity: 98.7%), α-cypermethrin (CAS: 67375-30-8, purity: 98.2%) and 3-phenoxybenzoic acid (CAS: 3739-38-6, purity: 98%), L-Thyroxine (T_4), prealbumin from human plasma (TTR) and the fluorescent probe 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANSA) were purchased from Sigma-Aldrich (Schnelldorf Distribution, Germany). Stock solutions of 75 mM 3-PBA and 200 mM deltamethrin, etofenprox and α-cypermethrin and 12.5 mM T_4 were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Schnelldorf Distribution, Germany). Chemical structures are shown in Figure 1. Stock solution of 30 mM ANSA was prepared in PBS. TTR was dissolved in PBS to reach a stock concentration of 17.27 µM.

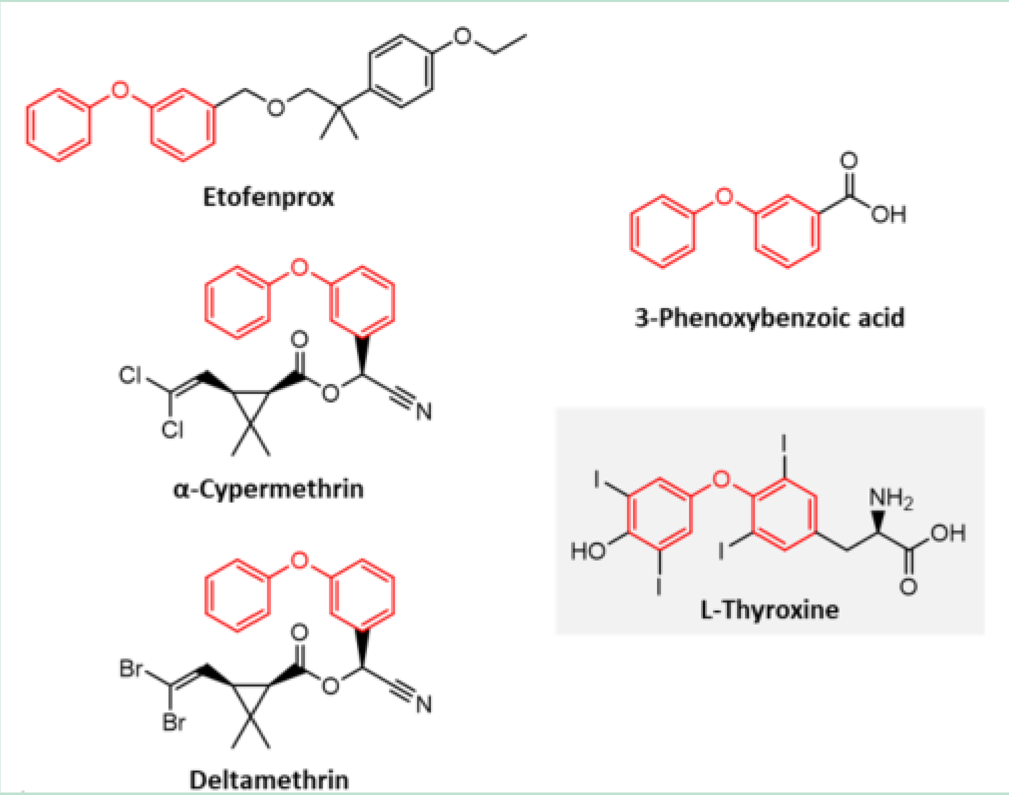

FIGURE 1. Chemical structure of the test substances

5.1.2 TTR binding *in vitro* - ANSA-TTR displacement assay.

Parent pyrethroids were tested at 2, 20 and 200 µM, the metabolite 3-PBA was tested at concentrations ranging from 0.049 to 200 µM, and vehicle (DMSO) was kept constant at 1% for all treatments. The ANSA-TTR displacement assay was performed as previously described in Rosenmai et al. (2021). Briefly, chemical dilutions, 0.6 µM ANSA and 0.5 µM TTR prepared in PBS were added to black flat-bottomed 96-well plates (PerkinElmer). A negative control with only ANSA, a positive control with ANSA-TTR and a displacement control of T_4 at concentrations of 0.2, 0.6 and 2.5 µM were included in each experiment. After 2 hours incubation at 4°C, plates were shaken for 15 seconds, and fluorescence was measured using an EnSpire microplate reader (PerkinElmer, Inc., Massachusetts, USA) with excitation at 380 nm and emission at 475 nm. Autofluorescence was tested by adding chemicals into ANSA solution without TTR. Three independent experiments were conducted with three technical replicates in each experiment.

5.1.3 Cardiomyocyte differentiation – the PluriBeat and PluriLum assay

The hiPSC BIONi010-C cell line (Bioneer A/S, Hørsholm, Denmark), which was previously genetically modified to incorporate a luciferase reporter under control of the cardiac-specific homeobox gene NKX2.5 (Lauschke et al., 2021), was cultured in mTeSR™1 medium (STEM-CELL Technologies, Vancouver, Canada), on hESC-Qualified Matrigel (Corning, New York, USA) coated cell culture dishes, and maintained at 37°C, at 5% CO2.

The cytotoxicity of deltamethrin, α-cypermethrin, etofenprox, and the metabolite 3-PBA was assessed in undifferentiated monolayers of human-induced pluripotent stem cells (hiPSCs) (Lauschke et al., 2020) in order to select non-cytotoxic concentrations for further assessments on embryonic bodies (EBs). For this purpose, single-cell suspensions of hiPSCs were seeded onto Matrigel-coated flat-bottom 96-well plates at a density of 1 × 104 cells/well and allowed to attach for 24 h. Medium was then replaced. After 24 h, cells were exposed to the test compounds, freshly prepared every 24 h, for a total of 48 h. Cytotoxicity of cell lysates was measured by the end of the exposure period through the CellTiter-Glo® 2.0 Cell Viability Assay (Promega, Wisconsin, USA). Three independent experiments were conducted, in 6 replicates, at different ranges of concentrations for each compound: 6.3 – 100 µM deltamethrin, 13 – 200 µM α-cypermethrin, 1.6 – 200 µM etofenprox, and 13 – 200 µM 3-PBA.

For the evaluation of the developmental effects of the test compounds, two assays were performed using single EBs: PluriBeat (Lauschke et al., 2020) and PluriLum (Lauschke et al., 2021) assays. Single-cell suspensions of hiPSCs were seeded onto Nunc™ 96-well polystyrene conical bottom MicroWell™ plates (Thermo Fisher Scientific, Massachusetts, USA) at a density of 5 × 104 cells/well in mTeSR™1 medium containing Rho Kinase inhibitor (Y-27632) and Penicillin-Streptomycin-Glutamine. Plates were centrifuged at 500g for 5 min, and incubated overnight at 37°C, at 5% CO^2 to allow the formation of the EBs. Medium was replaced after 24 h (D0). At D1, D2, D3 and D6, EBs were exposed to the pyrethroid compounds at a non-toxic concentration range (3.1 – 50 µM deltamethrin, 6.3 – 100 µM α-cypermethrin, 1.6 – 25 µM etofenprox, and 6.3 – 100 µM 3-PBA), which were prepared in medium containing the appropriate factors for cardiomyocyte differentiation. Three independent experiments were carried out with each treatment condition, in 10 replicates (i.e., 10 EBs). Following exposure, for the PluriBeat assay, each EB was observed for 10 sec under an inverted microscope and scored for ob-servable contractility. Beat scoring was performed as follows: 0 – if no movement was observed; 2 – if the entire area of the sphere contract-ed; 1 – everything in between. For the PluriLum assay, each EB was then transferred onto white 96-well plates for luminescence measurement using the Nano-Glo Live Cell Assay System (Promega, Wisconsin, USA).

5.1.4 Data processing and statistics

For the TTR-ANSA data, the fluorescence from the negative control was subtracted to all data, and results were presented as fluorescence relative to the maximum fluorescence produced by ANSA-TTR binding, set as 1. Data were analyzed using one-way ANOVA followed by a Dunnett's multiple comparison test using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). The lowest observable effect concentration (LOEC) was defined as the lowest tested concentration that is significantly different from the control.

For the cytotoxicity assay, means from independent experiments were pooled and exposure concentrations leading to more than 20% decreased cell viability was perceived as cytotoxic. PluriBeat data was analyzed through ordinal regression using IBM SPSS software version 27 for Windows (IBM, New York, USA). PluriLum data was analyzed using GraphPad Prism version 9 for Windows GraphPad Soft-ware, California, USA) through one-way ANOVA, followed by Dunnett's post-hoc test for multiple comparisons.

5.2 Epidemiological studies

5.2.1 Study population

The Odense child Cohort (OCC) is a prospective birth cohort established in Denmark. Pregnant women residing in Odense Municipality between 2010 and 2012 were recruited at first ultrasound scan (Kyhl et al., 2015). Briefly, all women living in the Municipality of Odense who were newly pregnant between 2010 and 2012 were invited to participate (N=6707) either at information meetings, or at the ultrasound examination conducted at Odense University Hospital between GW 10 and 16. Of the eligible women, 4017 accepted to receive information, and 2874 (43%) agreed to participate.

At enrolment in GW 10-15, the women were asked to respond to a questionnaire about general health, medication, and lifestyle, and to donate a fasting blood sample. Around GW 28, the women provided a urine sample. The samples were collected in the morning and stored at -80°C in the Odense Patient data Explorative Network (OPEN) biobank, until chemical analyses (Kyhl et al., 2015).

After the children were born, the families were invited to participate in clinical examinations of the children at age 3 months, 18 months, 3 years, 5 years, and 7 years and at each examination the parents were asked to complete a questionnaire on child health and development. At age 5 years, the children were also asked to provide a urine sample.

Information about covariates (maternal age, pre-pregnancy BMI, parity, smoking status, education level, birth weight and gestational age at birth) was obtained from questionnaires and hospital records. Information regarding duration of breastfeeding was obtained from the questionnaires filled in at child aged 3 and 18 months, as well as from a sub-project on breastfeeding reported via text messages (Bruun et al., 2016).

At the clinical examinations at age 18 months, and 3, 5, and 7 years, child height (to the nearest centimeter) was measured with a stadiometer and child weight (to the nearest 0.1 kg), with minimal clothing, was measured on a digital weight scale by trained staff professionals at OCC. Individual age- and sex-specific standard deviation scores for BMI (BMI Z-score) was calculated as described by Tinggaard et al. (2014) using Danish longitudinal growth data as reference.

For this project, multiple pregnancies were excluded. We included singleton mother-child pairs (N=2448) for whom we had available measurements of urinary pyrethroid metabolite concentrations from the mothers (N=1183) and/or children (N=1237). As the number of participating families varied from examination to examination, the number of participants in the individual sub-studies of e.g., neurodevelopment and blood pressure was lower and differed across the sub-studies.

5.2.2 Pyrethroid exposure assessment

Spot urine samples from mothers and children were analyzed for the generic pyrethroid metabolite, 3-PBA, representing the combined exposure to most pyrethroids. Maternal urine samples were also analyzed for the following specific pyrethroid metabolites: *cis*- and *trans*-DCCA (3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid) representing exposure to the *cis*- and *trans*-isomers of permethrin, cypermethrin, and cyfluthrin; 4-F-3PBA (4-fluoro-3-phenoxybenzoic acid), a specific metabolite of cyfluthrin; and *cis*-DBCA (cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid), a specific metabolite of deltamethrin. Urine samples from the children were analyzed for *trans*-DCCA and a specific metabolite of bifenthrin and λ-cyhalothrin, CFCA (3-(-2-Chloro-3,3,3-trifluoroprop-1-enyl)-2,2- dimethyl-cyclopropane-carboxylic acid). Furthermore, all urine samples were analyzed for a specific metabolite of chlorpyrifos/chlorpyrifos-methyl, TCPY (3,5,6-trichloro-2-pyridinol), was also included. The analyses were performed by high performance liquid chromatography and tandem mass spectrometry (LC-MS/MS) as previously described (Dalsager et al., 2019). Calibration curves, solvent blanks, and quality control samples were included in each batch of urine samples. In-house quality control (QC) samples (low and high level) with all compounds were made in diluted urine (1:3). The accuracy of the analysis was controlled by participation in the German External Quality Assessment Scheme (G-EQUAS) for 3-PBA, trans-DCCA, and cis-DBCA. Excess sample material from this program was also used as QC samples. The accuracy of the analysis for all the compounds during the series of samples analysed ranged from 85.0 - 94.2%. The between batch variation (CV%) ranged from 5.9 - 18.8%. The limits of detection (LOD) for the compounds were: 0.03 µg/L for 3-PBA; 0.2 µg/L for 4-F-3PBA; 0.4 µg/L for trans-DCCA, 0.5 µg/L for cis-DCCA and cis-DBCA, and 0.3 µg/L for TCPY.

To enable adjustment for urine dilution, spectrophotometric determination of creatinine concentrations in the urine samples was included. These analyses were conducted on a Konelab 20 Clinical Chemistry Analyzer, using a commercial kit (Thermo, Vantaa, Finland). Seronorm Urine (L1 and L2) from Sero (Sero AB, Billingstad, Norway) was included as QC samples with each series of samples. The accuracy of the analysis was controlled by regular participation in the G-EQUAS programme. Excess sample material from this program was also included with each series of samples as QC samples. The between batch variation (CV%) was < 7.0%. The accuracy of the analysis ranged from 99.7-108.7%.

All analyses were performed at Clinical Pharmacology, Pharmacy and Environmental Medicine, Department of Public Health, University of Southern Denmark.

5.2.3 Neurodevelopmental outcomes

5.2.3.1 Child Behavior Checklist - ADHD and ASD scores

The Child Behavior Checklist (CBCL) is a worldwide frequently used validated and standardized parent-reported questionnaire of children´s behavioral and emotional problems, with solid psychometric properties (Aebi et al., 2010; Kristensen et al., 2010; Skarphedinsson et al., 2021). In the OCC, ADHD and Autism Spectrum Disorder (ASD) symptoms at age 2-3 and 5-6 years were assessed using the CBCL for preschool children: CBCL/1½-5 (Achenbach and Rescorla, 2000). The CBCL/1½-5 consists of 100 problem items, which are grouped into several scales and subscales. Respondents are requested to rate each item based on the preceding two months, as 0 for not true, 1 for somewhat or sometimes true and 2 for very true or often true, reflecting a 3-point Likert scale. A total problem scale as well as five problem scales are computed including an “ADHD problem scale” and an “PDP problem scale” (Kristensen et al., 2010). The “ADHD scale” is based on 6 items with a total score range of 0-12 points (Table 1) and the “PDP problem scale” is based on 13 items with a total score range of 26 (Table 2). The families were invited to complete the questionnaire online.

TABLE 1. CBCL/1½-5 ADHD problem scale

Items	Not true	Somewhat/ sometimes true	Very often/often true
#5 Cannot concentrate, can´t pay attention for long	0	1	2
#6 Cannot sit still, restless, or hyperactive	0	1	2
#8 Cannot stand waiting, wants everything now	0	1	2
#16 Demands must be met immediately	0	1	2

#36 Gets into everything	0	1	2
#59 Quickly shifts from one activity to another	0	1	2
ADHD problem score		0-12	

TABLE 2. CBCL/1½-5 Pervasive Developmental Problem (PDP) scale (Autism Spectrum Disorder scale)

Items	**Not true**	**Somewhat/ sometimes true**	**Very often/often true**
#3. Afraid to try new things	0	1	2
#4. Avoids looking others in the eye	0	1	2
#7. Can't stand things out of place	0	1	2
#21. Disturbed by any change in routine	0	1	2
#23. Doesn't answer when people talk to him/her	0	1	2
#25. Doesn't get along with other children	0	1	2
#63. Repeatedly rocks head or body	0	1	2
#67. Seems unresponsive to affection	0	1	2
#70. Shows little affection toward people	0	1	2
#76. Speech problems	0	1	2
#80. Strange behavior	0	1	2
#92. Upset by new people or situations	0	1	2
#98. Withdrawn, doesn't get involved with others	0	1	2
PDP/ASD problem score		0-26	

5.2.3.2 Cognitive function - Intelligence quotient (IQ)

Children were invited for assessment of cognitive function at their school two weeks prior to their 7th birthday. An abbreviated, less time-consuming version of the Danish Wechsler Intelligence Scale for Children fifth edition (WISC-V) (Wechsler, 2017) consisting of four subtests (out of the standard seven): vocabulary, similarities, block design and matrix reasoning was used (Beck et al., 2023). Four trained psychologists performed the assessments. The testing was primarily performed by one psychologist (tester 1) and assisted by three other psychologists (tester 2-4). An experienced psychologist acted as a supervisor for the four psychologists.

The WISC-V is the most validated tool to estimate IQ in children aged 6.0 to 16.9 years (Wechsler, 2017). From the four WISC-V sub-tests included in the OCC study, a full-scale IQ (FSIQ) was estimated, and this four subtests FSIQ score has been shown to be a valid predictor for the seven subtests FSIQ score (Beck et al., 2023). In addition, a verbal comprehension index (VCI) was calculated based on two of the subtests (vocabulary and similarities). Both the FSIQ- and VCI-scores have a have a standardizes mean (standard deviation (SD)) of 100 (15) points based on age-appropriate IQ scores from the Danish background population (Wechsler, 2017).

5.2.4 Maternal Thyroid hormones

Serum obtained from fasting blood samples collected at enrolment were analyzed for thyroid stimulating hormone (TSH), free thyroxine (fT4), free triiodothyronine (fT3) and thyroid peroxidase antibody (TPOab). The analyses were performed using an electrochemiluminescence immunoassay on the E801 module at the Roche Cobas 8000 platform (Roche Diagnostics

GmbH) as previously described (Andersen et al., 2021b). Positive TPOab was defined as values above 34 kIU/L (Andersen et al., 2021b).

5.2.5 Blood pressure measurement

Systolic and diastolic blood pressure (SBP/DBP) were measured in children at age 8 months, and at 3, 5, and 7 years. All measurements were performed by trained technicians using an automatic sphygmomanometer (Welch Allyn) on the upper left arm with cuffs of appropriate sizes and with the child resting in a sitting position.

5.2.6 Ethics

OCC based studies included in this project were performed in accordance with the second Helsinki Declaration, with written, informed consent, and approved by The Regional Committees on Health Research Ethics for Southern Denmark (S-20090130) and the Danish Data Protection Agency.

5.2.7 Data processing and statistics

For the insecticide metabolites, values below the limit of detection (LOD) were substituted by the metabolite specific LOD/√2. Most of the specific pyrethroid metabolites (4-F-3PBA, *cis*-DCCA, *cis*-DBCA, and CFCA) had detection frequencies below 10% (Table 3) and were therefor not included in further statistical analyses. *Trans*-DCCA was included in some statistical analyses as a dichotomized variable (≥LOD vs <LOD). Urinary concentrations of 3-PBA and TCPy were not normally distributed and were therefore reported as medians and percentiles. Bivariate associations between urinary 3-PBA and TCPy concentrations and population characteristics were analyzed with Kruskal Wallis Test. For further analyses, the continuous concentrations were either log-transformed to obtain a normal distribution or categorized into tertiles. Associations between urinary metabolites and health outcomes were analyzed using different types of regression models depending on data type and distribution as described below. Potential confounding variables were selected *a priori* based on review of the literature and bivariate associations seen in our data (Table 4). In all analyses, creatinine (g/L) was included as a covariate to adjust for dilution of the urine samples.

The CBCL/1½-5 ADHD- and ASD-scores were right skewed and considered as count data. The score data was dichotomized to indicate a score equal to or above the 90th percentile for the included children. Associations between maternal and child insecticide metabolite concentrations and the dichotomized ADHD/ASD variables were analyzed using logistic regression. The estimates were presented as odds ratios (OR) for scoring ≥ the 90th percentile on the ADHD/ASD problem scales compared to the low tertile or the relative change for a doubling in the urinary metabolite concentration for the log-transformed variable. A Z-test for a linear trend across the exposure tertile groups was also conducted. Maternal educational level, as the best available estimate of maternal IQ and socio-economic status, as well as parental psychiatric diagnosis were identified as potential confounders and were adjusted for in the regression models. Further, since symptoms of ADHD strongly depends on age and sex, child age at examination and child sex were also included in the regression analyses.

The FSIQ- and VCI-scores were normally distributed and reported as mean and standard deviation (SD). Associations between maternal and child insecticide metabolite concentrations and FSIQ/VCI-scores were investigated by linear regression. The estimates were presented as change in IQ-points compared to the lowest tertile or per doubling of the urinary metabolite concentrations for the log-transformed variable. The regression models were adjusted for maternal education level (as the best available estimate of socio-economic status and maternal IQ) and child sex.

All regression analyses of the neurodevelopmental outcomes were conducted both for the children together and stratified by child sex as cognitive function and many behavioral disorders, including ADHD and ASD, present with a strong sex-bias during childhood (Beck et al., 2023; Mowlem et al., 2019; Napolitano et al., 2022). We also checked for interactions between exposure and child sex by including an interaction (multiplication) term in the regression models.

Serum concentrations of TSH, fT4, and fT3 were non-normally distributed and reported as median and percentiles, and log-transformed to obtain normally distributed residuals and variance homogeneity. The estimates from linear regression analyses were transformed to express percent change in TH concentrations compared to the lowest exposure tertile. Pre-pregnancy BMI, smoking, age, and socioeconomic status (SES) were identified as factors that may affect TH hormones in pregnancy (Andersen et al., 2021; Mehran et al., 2019). We adjusted for pre-pregnancy BMI (continuous) and smoking in pregnancy (yes/no), as these variables were related to 3-PBA in our data set (Table 4). Age and maternal education level (as proxy for SES) were unrelated to 3-PBA but were included in sensitivity analyses. TPO is the primary enzyme involved in thyroid hormone production, and the presence of TPO antibodies (TPOab) may inhibit TPO. Potential effect modification by TPOab status on the association between 3-PBA and thyroid hormones was investigated by repeating the regression analyses stratified to TPO-ab status and by including an interaction term between 3-PBA tertiles and TPOab status in the regression models.

Blood Pressure (BP) data were normally distributed and reported as mean and SD. Mean differences in BP according to population characteristics and between exposure tertile groups were tested by ANOVA. Associations between maternal and childhood pyrethroid exposure and systolic BP (SBP) and diastolic BP (DBP) during childhood were analyzed using multiple linear regression models, adjusting for age-and-sex specific BMI z-scores, child sex, and urinary creatinine. Effect modification by sex was analyzed by including an interaction term (multiplication of sex and exposure variables). In sensitivity analyses, we further included maternal age, BMI, and smoking status in pregnancy in the regression models for prenatal exposure.

6. Results

6.1 Experimental studies

6.1.1 Binding to transthyretin (TTR)

The pyrethroid metabolite 3-PBA significantly displaced ANSA from TTR (LOEC = 1.6 µM; $p < 0.001$ versus control) (Figure 2). None of the pyrethroids were able to bind to TTR at the tested range. Etofenprox displayed a significant increase of fluorescence at the highest test concentration of 200 µM ($p < 0.01$ versus control), which was proven to be due to autofluorescence of the chemical.

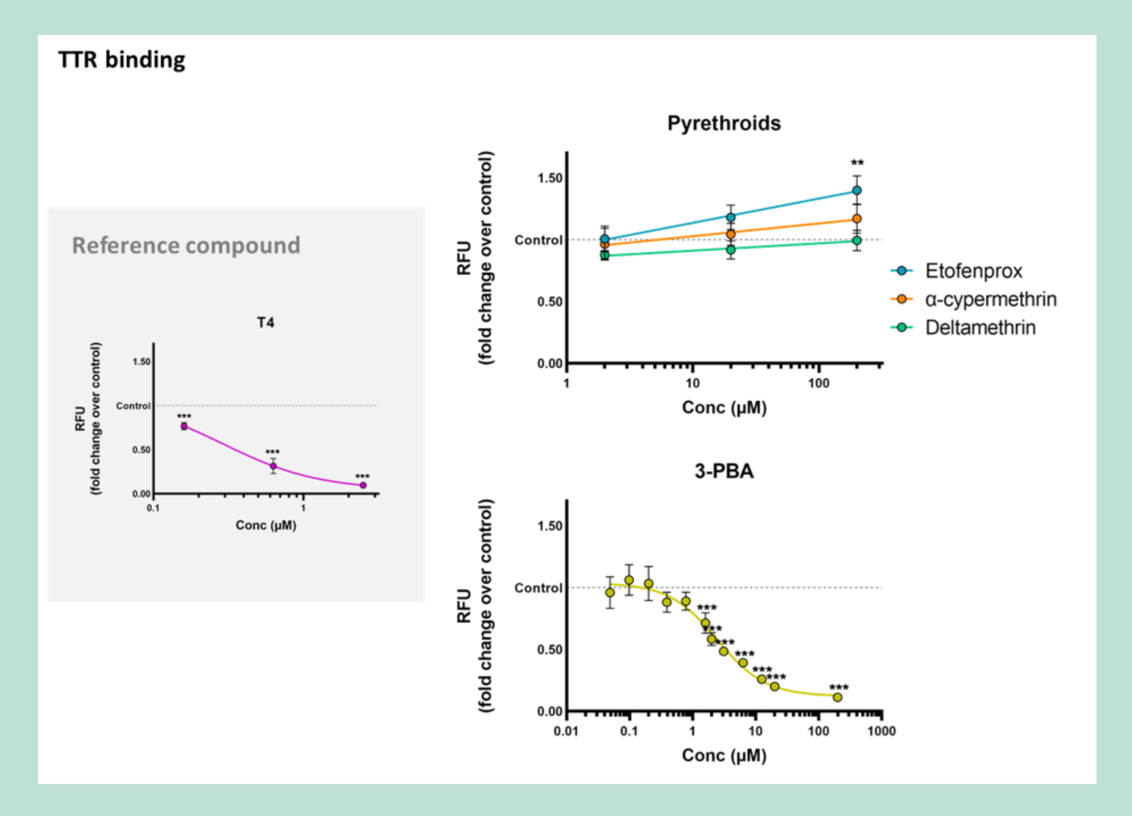

FIGURE 2. TTR binding potential of L-thyroxine (T4) as a displacement control, pyrethroids and the common metabolite 3-phenoxybenzoic acid (3-PBA). Results are from three independent experiments, performed in triplicates. Data are presented as mean ± SD. ** $p < 0.01$, ***$p < 0.001$ versus control.

6.1.2 Cardiomyocyte differentiation

Regarding the cytotoxicity assay performed in undifferentiated hiPSCs, etofenprox showed a decrease in cell viability from 50 µM, deltamethrin from 100 µM, while 3-PBA and α-cypermethrin showed no cytotoxicity at any concentration tested (data not shown).

As depicted in Figure 3, only α-cypermethrin showed effects on the PluriBeat assay when compared to control within a non-cytotoxic range of concentrations, inducing a significant decrease in beat score from 50 µM ($p < 0.001$ versus control).

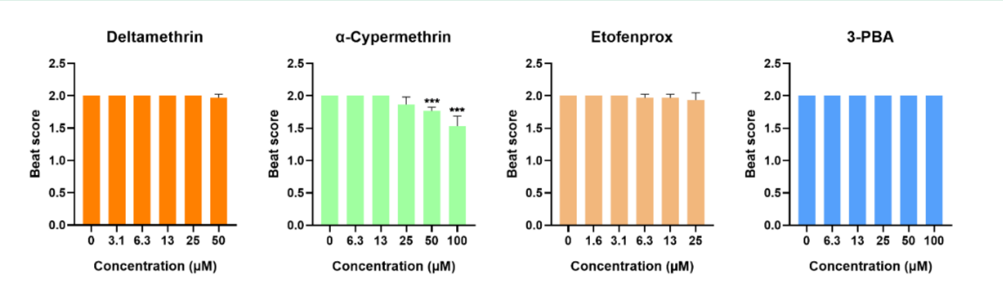

FIGURE 3. Beat score assessment of embryoid bodies exposed for 6 days to increasing concentra-tions of three pyrethroid insecticides and the metabolite 3-phenoxybenzoic acid (3-PBA). Results are from three independent experiments, performed in ten replicates. Data are presented as mean ± SD. ***p < 0.001 versus control.

On the other hand, all three parent pyrethroid insecticides, but not the metabolite 3-PBA, were capable of inducing a concentration-dependent decline in luminescence in the PluriLum assay (Figure 4). This effect was significant from 13 µM for deltamethrin (p < 0.01 versus control), 6.3 µM for α-cypermethrin (p < 0.05 versus control), and already from 1.6 µM for etofenprox (p < 0.05 versus control), with a maximum fold decrease over control of around 37 % for deltamethrin, 42% for α-cypermethrin, and 22% for etofenprox (p < 0.001 for all three com-pounds versus control) at the highest concentration tested.

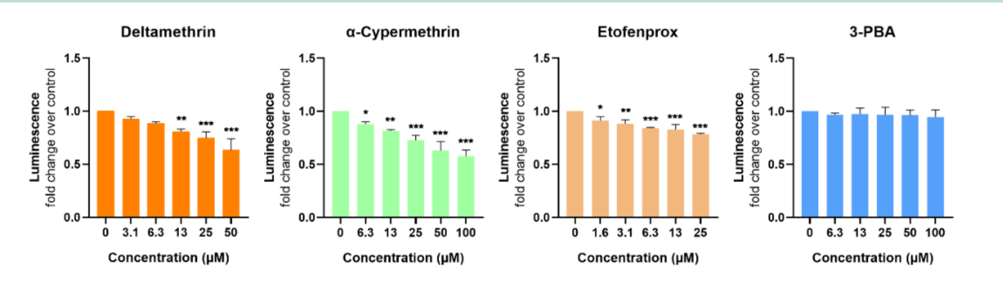

FIGURE 4. Luminescence assessment of embryoid bodies exposed for 6 days to increasing concentra-tions of three pyrethroid insecticides and the metabolite 3-phenoxybenzoic acid (3-PBA). Results are from three independent experiments, performed in ten replicates. Data are pre-sented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus control.

6.2 Epidemiological studies from the Odense Child Cohort (OCC)

6.2.1 Study population and exposure level

Urinary metabolite concentrations of pyrethroids and chlorpyrifos were available from 1207 mothers and 848 singleton children before this project was initiated. As part of this project, another 389 urine samples from 5 years-old singleton children were selected, based on availability of health outcome data, and analyzed. We excluded 24 mothers who gave birth to twins. Thus, 1183 mothers and 1237 children with urinary insecticide metabolite concentrations were included in this project.

When the first batches of child urine samples were analyzed in 2021, it turned out that almost no samples had detectable concentrations of 4-F-3PBA, *cis*-DCCA, or *cis*-DBCA. Therefore, it was decided to skip these metabolites. Instead, a more recent developed biomarker for bifenthrin and λ-cyhalothrin, CFCA, was included. However, the detection rate of this biomarker as well as most other specific pyrethroid biomarkers were in general low and only *trans*-DCCA was detectable in more than 10% of the urine samples (Table 3). The generic pyrethroid metabolite, 3-PBA, had a lower LOD and was detectable in most, i.e., 93.7 % and 89.7 % of the samples from mothers and children, respectively. Also, the chlorpyrifos metabolite, TCPy, was detectable in most urine samples. 3-PBA and trans-DCCA concentrations were positively correlated for both mothers (spearman's rho = 0.42) and children (spearman's rho = 0.32). Also, TCPy and 3-PBA were positively correlated for both mothers and children (spearman's rho = 0.40 for both). Children had lower concentrations than the mothers of 3-PBA (median: 0.18 vs 0.21 µg/L) and TCPy (median: 1.15 vs 1.65 µg/L) and the concentrations were only weakly correlated (spearman's rho=0.04 for 3-PBA and 0.05 for TCPy).

Most of the included mothers were non-smokers (96.7%) and nulliparous (56.0%) and had a BMI in the normal range (60.4%) and approximately half of the women (50.5%) had an intermediate education level (Table 4). Women with high BMI and short duration of breastfeeding had higher urinary 3-PBA concentration. Older and well-educated women tended to have higher TCPy concentrations. A higher median TCPy concentration was also seen among children with birthweight above 3500 g compared to those below.

TABLE 3. Urinary concentrations of pyrethroid and chlorpyrifos metabolites among 1183 mothers (gestational week 28) and 1237 children (age 5 years) from the Odense Child Cohort

Parent compound	LOD	Maternal urinary concentrations, µg/L						Child urinary concentrations, µg/L					
		%≥LOD	P25	P50	P75	P95	max	%≥LOD	P25	P50	P75	P95	Max
most pyrethroids (*not* cyfluthrin or bifenthrin)	0.03	93.7	0.10	0.21	0.49	1.95	75.96	89.7	0.08	0.18	0.37	1.30	38.4
cyfluthrin	0.2	0.1	<LOD	<LOD	<LOD	<LOD	2.90	NI	NI	NI	NI	NI	NI
trans- permethrin, -cyperme-thrin, -cyfluthrin	0.4	12.3	<LOD	<LOD	<LOD	1.42	26.27	11.5	<LOD	<LOD	<LOD	0.92	28.62
cis-permethrin, -cypermethrin, -cyfluthrin	0.5	2.7	<LOD	<LOD	<LOD	<LOD	20.07	NI	NI	NI	NI	NI	NI
deltamethrin	0.5	2.5	<LOD	<LOD	<LOD	<LOD	1.97	NI	NI	NI	NI	NI	NI
bifenthrin, λ-cyhalothrin	0.2	NI	NI	NI	NI	NI	NI	7.7	<LOD	<LOD	<LOD	0.33	4.83
chlorpyrifos, chlorpyrifos-methyl	0.3	90.4	0.81	1.65	3.13	8.42	65.91	83.3	0.58	1.15	2.21	5.41	131.4

LOD: limit of detection; NI: not included; p25-p95: 25th-95th percentiles; 3-PBA: 3-phenoxybenzoic acid; 4F-3-PBA: 4-fluoro-3-phenoxybenzoic acid; *cis*-DCCA: *cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid; *trans*-DCCA: *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid; *cis*-DBCA: cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid; CFCA (3-(-2-Chloro-3,3,3-trifluoroprop-1-enyl)-2,2- dimethyl-cyclopropane-carboxylic acid; TCPy: 3,5,6-trichloro-2-pyridinol.

TABLE 4. Urinary concentrations (µg/L) of 3-PBA and TCPy among mothers and children (age 5 years) according to study population characteristics

	Maternal			Child		
Characteristics	N (%)	M (p25-p75)		N (%)	M (p25-p75)	
		3-PBA	TCPy		3-PBA	TCPy
All	1183 (100)	0.21 (0.10-0.49)	1.65 (0.81-3.13)	1237 (100)	0.18 (0.08-0.37)	1.15 (0.58-2.21)
Maternal age at birth (years)						
<30	510 (43.1)	0.20 (0.10-0.50)	**1.64 (0.78-3.32)**	496 (40.1)	0.18 (0.08-0.39)	1.14 (0.56-2.19)
30-35	452 (38.2)	0.21 (0.10-0.49)	**1.52 (0.78-2.83)**	495 (40.0)	0.18 (0.08-0.37)	1.15 (0.56-2.22)
>35	221 (18.7)	0.23 (0.10-0.47)	**2.02 (1.00-3.51)**	245 (19.8)	0.16 (0.07-0.34)	1.18 (0.66-2.24)
Maternal educational level						
High school or less	336 (28.4)	0.23 (0.10-0.51)	1.50 (0.80-2.84)	317 (25.6)	0.19 (0.08-0.39)	1.20 (0.58-2.23)
High school + 1-4 years	598 (50.5)	0.20 (0.10-0.49)	1.68 (0.82-3.21)	626 (50.6)	0.17 (0.08-0.34)	1.13 (0.58-2.18)
High school + 5 or more years	238 (20.1)	0.20 (0.10-0.47)	1.86 (0.86-3.41)	270 (21.8)	0.18 (0.08-0.41)	1.11 (0.57-2.26)
Missing information	11 (0.9)	0.52 (0.11-0.73)	0.85 (0.21-2.48)	24 (1.9)	0.14 (0.03-0.30)	0.95 (0.28-2.34)
Maternal pre-pregnancy BMI (kg/m^2)						
<18.5	32 (2.7)	**0.17 (0.06-0.29)**	2.07 (0.71-3.43)	37 (3.0)	0.20 (0.08-0.32)	1.37 (0.45-2.66)
18.5-25	715 (60.4)	**0.19 (0.09-0.42)**	1.59 (0.78-3.08)	774 (62.6)	0.18 (0.07-0.37)	1.12 (0.56-2.21)
>25	436 (36.9)	**0.25 (0.12-0.69)**	1.72 (0.88-3.22)	425 (34.4)	0.18 (0.09-0.37)	1.18 (0.59-2.17)
Maternal parity						
0	663 (56.0)	0.21 (0.10-0.50)	1.60 (0.75-3.21)	656 (53.1)	0.18 (0.08-0.38)	1.08 (0.55-2.14)
>0	520 (44.0)	0.20 (0.10-0.48)	1.70 (0.89-3.07)	580 (46.9)	0.17 (0.08-0.36)	1.24 (0.60-2.26)
Maternal smoking in pregnancy						
No	1144 (96.7)	0.20 (0.10-0.49)	1.65 (0.81-3.13)	1186 (96.1)	0.18 (0.08-0.36)	1.15 (0.58-2.22)

	Maternal			Child		
Yes	39 (3.3)	0.38 (0.10-0.87)	1.77 (0.69-3.81)	48 (3.9)	0.25 (0.09-0.63)	1.16 (0.66-1.99)
Breastfeeding (months)						
< 6	395 (33.4)	**0.26 (0.11-0.51)**	1.70 (0.79-3.35)	394 (31.9)	0.18 (0.08-0.35)	1.14 (0.57-2.07)
≥ 6	522 (44.1)	**0.20 (0.10-0.49)**	1.65 (0.82-3.18)	590 (47.7)	0.17 (0.08-0.35)	1.12 (0.58-2.22)
Missing information	266 (22.5)	**0.17 (0.08-0.46)**	1.61 (0.84-3.03)	253 (20.5)	0.19 (0.08-0.43)	1.23 (0.58-2.46)
Child Sex						
Boy	622 (52.6)	0.21 (0.10-0.53)	1.63 (0.78-3.11)	713 (57.7)	0.20 (0.09-0.40)	1.21 (0.61-2.28)
Girl	561 (47.4)	0.21 (0.10-0.48)	1.67 (0.85-3.24)	523 (42.3)	0.15 (0.06-0.33)	1.06 (0.53-2.10)
Birthweight (grams)						
<3500	552 (46.7)	0.22 (0.11-0.49)	1.65 (0.78-3.04)	550 (44.5)	0.16 (0.08-0.36)	**1.03 (0.53-2.13)**
≥3500	631 (53.3)	0.20 (0.09-0.49)	1.66 (0.84-3.26)	686 (55.5)	0.19 (0.08-0.39)	**1.24 (0.61-2.26)**
Preterm birth < 37 gestational weeks						
No	1138 (96.4)	0.21 (0.09-0.49)	1.62 (0.81-3.11)	1185 (96.2)	0.18 (0.08-0.37)	1.15 (0.58-2.21)
Yes	43 (3.6)	0.24 (0.11-0.53)	2.20 (1.17-3.71)	47 (3.8)	0.14 (0.08-0.34)	1.29 (0.54-2.49)

M: median (=p50); p25-p75: 25th-75th percentiles; bold indicates statistically significant difference (p<0.05) between groups (Kruskal Wallis Test)

6.2.2 Exposure and neurodevelopment

6.2.2.1 ADHD and ASD scores

In a previous study based on the OCC, we found a significant association between maternal pyrethroid metabolites (3-PBA and trans-DCCA) in pregnancy and higher ADHD scores among the children at age 2-4 years (Dalsager et al., 2019). At follow-up when the children were 5 years of age, no significant associations between ADHD scores above the 90th percentile and maternal or child insecticide metabolite concentrations were observed (Table 5). Similar results were seen in sex-stratified analyses and accordingly no significant interactions between exposure and child sex were seen (results not shown). The 90th percentile ADHD scale score was 5 in both age groups and for both boys and girls.

TABLE 5. Adjusted Odds Ratio (OR) and 95% confidence interval (95% CI) for scoring > the 90th percentile on the CBCL ADHD problem scale according to maternal (gestational week 28) or child (age 5 years) urinary insecticide metabolite concentrations among full-term singleton mother-child pairs in the Odense Child Cohort.

	Prenatal exposure		Child exposure
	ADHD score at age 2-4 y[b]	ADHD score at age 5	ADHD score at age 5
N	936	614	814
3-PBA tertiles			
Low	Ref	Ref	Ref
Medium	1.09 (0.70; 1.71)	1.41 (0.78; 2.54)	0.72 (0.42; 1.22)
High	**1.90 (1.19; 3.05)**	1.29 (0.68; 2.43)	0.83 (0.48; 1.43)
Continuous[c]	**1.13 (1.01; 1.25**	1.01 (0.88; 1.16)	0.97 (0.85; 1.11)
Trans-DCCA			
≤LOD	Ref	Ref	Ref
>LOD	**1.76 (1.08; 2.86)**	0.81 (0.40; 1.65)	0.95 (0.51; 1.76)
TCPy			
Low	Ref	Ref	Ref
Medium	0.95 (0.62; 1.46)	1.47 (0.82; 2.63)	1.30 (0.77; 2.21)
High	0.92 (0.58; 1.45)	1.10 (0.58; 2.08)	1.39 (0.81; 2.41)
Continuous[c]	0.96 (0.85; 1.09)	1.04 (0.89; 1.23)	1.13 (0.98; 1.31)

[a]Adjusted for urinary creatinine, maternal education, parental psychiatric diagnosis, and child sex and age at examination; [b]Results from Dalsager et al. (2019); [c]OR when doubling the urinary metabolite concentration (log-transformed data); bold indicate p<0.05.

The number of children with available ADHD scores and maternal exposure data was lower at 5 years (N=614) than at 2-4 years (N=936). Of these, 560 children (60%) were included at both examinations, but the fraction of participating children was lower in the two upper 3-PBA tertiles than in the low tertile (Table 6). At age 2-4 years, 71 children in the high 3-PBA tertile had an ADHD score ≥ the 90th percentile. Of these 71 children, 15 (21%) scored ≥ the 90th percentile also at age 5 years, 24 (34%) scored below the 90th percentile, and 32 (45%) did not participate at age 5 years.

TABLE 6. Number and percentage, N (%), of children who scored < or > the 90th percentile (p90) on the CBCL ADHD problem scale at age 2-4 years and 5 years according to maternal 3-PBA tertiles in the study sample at child aged 2-4 years.

3-PBA tertiles	ADHD scores at age 2-4 years		ADHD scores at age 5 years		Both exam.
	N (%)< p90	N (%)≥ p90	N (%)< p90	N (%)≥ p90	N (%)
Low	263 (84.9)	47 (15.1)	179 (87.3)	26 (12.7)	205 (66)
Medium	268 (84.5)	49 (15.5)	147 (82.6)	31 (17.4)	178 (56)
High	238 (77.0)	71 (23.0)	146 (82.5)	31 (17.5)	177 (57)
Total	769 (82.1)	167 (17.9)	472 (84.3)	88 (15.7)	560 (60)

CBCL ASD scores at age 2-4 years were obtained from 998 children with available maternal insecticide metabolite concentrations (Table 7). The 90th percentile on the ASD scale score was 4 for both girls and boys and 164 (16.4%) of the children scored 4 or higher. We did not find any associations between maternal pyrethroid metabolite concentrations in pregnancy and the risk of scoring above the 90th percentile on the ASD-score scale, neither among all the children or in sex-stratified analyses.

For the chlorpyrifos metabolite, TCPy, the middle tertile of maternal concentrations was associated with higher odds of scoring above the 90th percentile among girls, but not among boys. No associations were seen for TCPY in the highest tertile.

TABLE 7. Adjusted Odds Ratio (OR) and 95% confidence interval (95% CI) for scoring > the 90th percentile on the CBCL ASD problem scale at age 2-4 years according to maternal urinary insecticide metabolite concentrations among full-term singleton mother-child pairs in the Odense Child Cohort.

	All	**Girls**	**Boys**
N	998	477	521
3-PBA tertiles	OR (95% CI)	OR (95% CI)	OR (95% CI)
Low	Ref	Ref	Ref
Medium	0.93 (0.60; 1.44)	1.13 (0.56; 2.28)	0.80 (0.46; 1.41)
High	0.99 (0.62; 1.61)	1.05 (0.48; 2.34)	1.00 (0.55; 1.83)
Continuous[b]	1.02 (0.92; 1.14)	1.05 (0.87; 1.26)	1.02 (0.90; 1.16)
Trans-DCCA			
≤LOD	Ref	Ref	Ref
>LOD	1.07 (0.63; 1.82)	0.58 (0.19; 1.72)	1.41 (0.75; 2.66)
TCPy			
Low	Ref	Ref	Ref
Medium	1.38 (0.89; 2.14)	**2.46 (1.18; 5.15)**	0.97 (0.55; 1.69)
High	0.96 (0.59; 1.56)	1.10 (0.46; 2.60)	0.92 (0.51; 1.67)
Continuous[b]	0.97 (0.86; 1.10)	0.97 (0.79; 1.19)	0.98 (0.84; 1.14)

[a]Adjusted for urinary creatinine, maternal age and education, parental psychiatric diagnosis, and child sex and age at examination; [b]OR when doubling the urinary metabolite concentration (log-transformed data); bold indicate p<0.05.

As no associations were seen between pyrethroid exposure and autism scores at age 2-4 years, we did not include the autism scores obtained at age 5 years in the data-analyses.

6.2.2.2 Cognitive function - Intelligence quotient (IQ)

IQ-testing at age 7 years was completed for 1510 children in the OCC of whom 818 had available data on maternal insecticide metabolite concentration. We excluded 6 women with missing information on education level. Thus, 812 mother-child pairs were included in the analyses of associations between prenatal pyrethroid exposure and cognitive function at school-age. The mean FSIQ score for the included children was 99.4, and it was higher for girls (100.6) than boys (98.4).

As seen from Figure 5, maternal pyrethroid metabolites were in general associated with lower FSIQ and VCI scores and the associations seemed stronger in girls than boys. However, none of the associations were statistically significant.

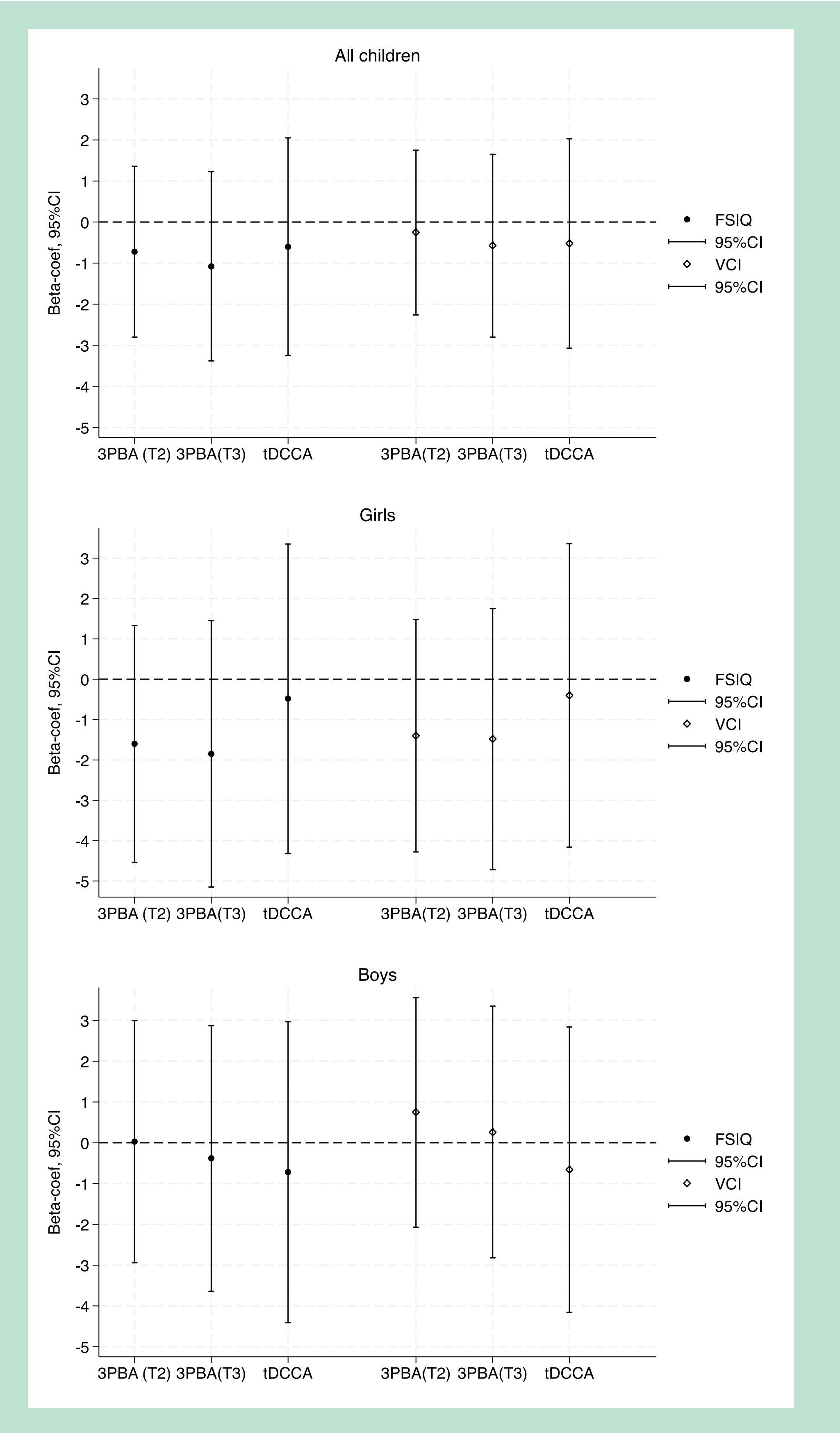

FIGURE 5. Adjusted results from linear regression analyses of associations between maternal pyrethroid metabolites and intelligence quotients at age 7 year among 812 children (395 girls and 417 boys) from the Odense Child Cohort. The Beta-coefficients present change in Full-

Scale Intelligence Quotient (FSIQ) and Verbal Comprehension Index (VCI) scores with 95% confidence intervals (95% CI) for maternal 3-PBA concentrations in the 2. (T2) or 3. (T3) tertile compared to the first tertile (T1), and for trans-DCCA > LOD compared to < LOD. The models were adjusted for maternal education and urinary creatinine and child sex (all children).

Prenatal chlorpyrifos exposure (maternal TCPy) was also associated with reduced FSIQ scores (Figure 6) and VCI scores (not shown), although only significant for the middle tertile, and only among girls. To investigate whether this association was influenced by potential residual confounding from socioeconomic factors, the data analyses were repeated for low educated (high school or less) mothers only. These results showed a dose-related reduction in FSIQ scores across TCPy tertiles for both girls and boys, but the associations had wide confidence intervals due to the smaller sample size and did not reach statistical significance.

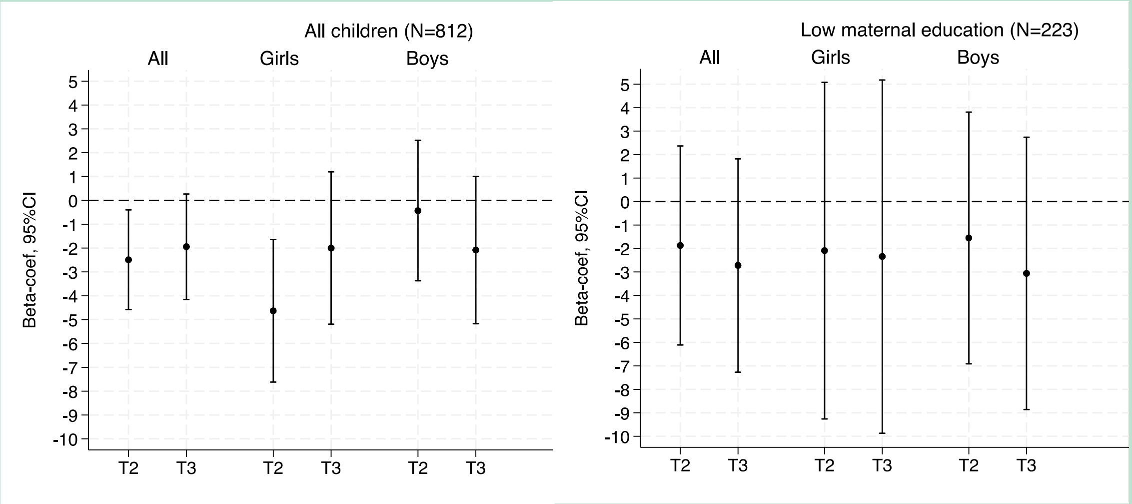

FIGURE 6. Adjusted results from linear regression analyses of associations between maternal TCPy (chlorpyrifos exposure) and Full-Scale Intelligence Quotient (FSIQ) at age 7 year among 812 children (395 girls and 417 boys) and among 223 children (90 girls and 133 boys) of low-educated mothers (right figure) from the Odense Child Cohort. The Beta-coefficients present change in FSIQ scores with 95% confidence intervals (95% CI) for maternal TCPy concentrations in the 2. (T2) or 3. (T3) tertile compared to the first tertile (T1). The models were adjusted for maternal education and urinary creatinine and child sex (all children).

Regarding childhood exposure, we did not find any associations between urinary concentrations of pyrethroid metabolites at age 5 years and FSIQ or VCI scores at age 7 years (Figure 7) among 1071 children with available data for both exposure and outcome.

In contrast, clear inverse associations were seen between urinary TCPy concentrations at age 5 years and FSIQ scores at age 7 years (Figure 8). The association was stronger among girls than boys. Further adjustment for length of breastfeeding did not materially affect the results.

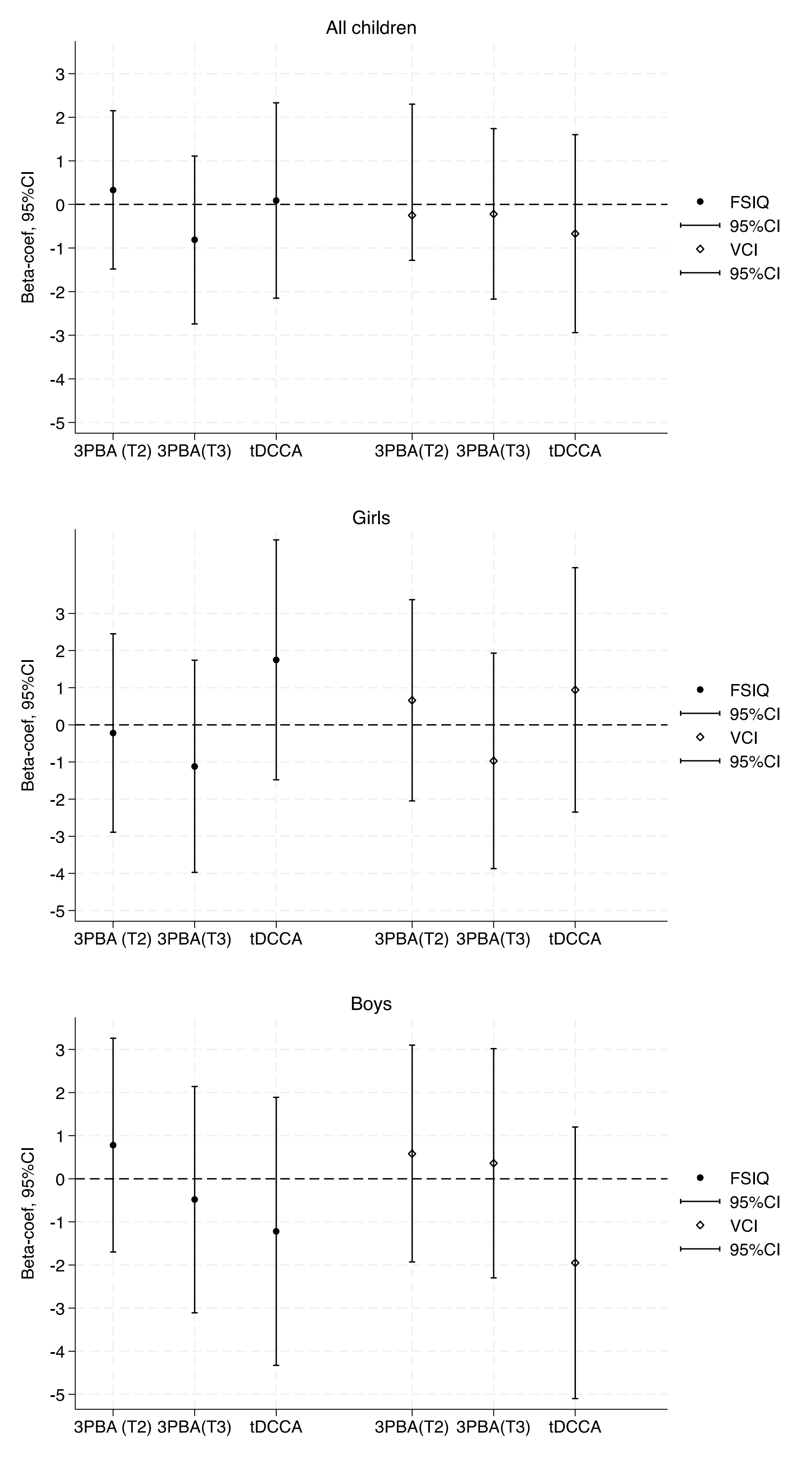

FIGURE 7. Adjusted results from linear regression analyses of associations between child pyre-throid metabolites and intelligence quotients at age 7 year among 1071 children (460 girls and 611 boys) from the Odense Child Cohort. The Beta-coefficients present change in Full-

Scale Intelligence Quotient (FSIQ) and Verbal Comprehension Index (VCI) scores with 95% confidence intervals (95% CI) for child 3-PBA concentrations in the 2. (T2) or 3. (T3) tertile compared to the first tertile (T1), and for trans-DCCA > LOD compared to < LOD. The models were adjusted for maternal education and for child sex (all children) and urinary creatinine concentration.

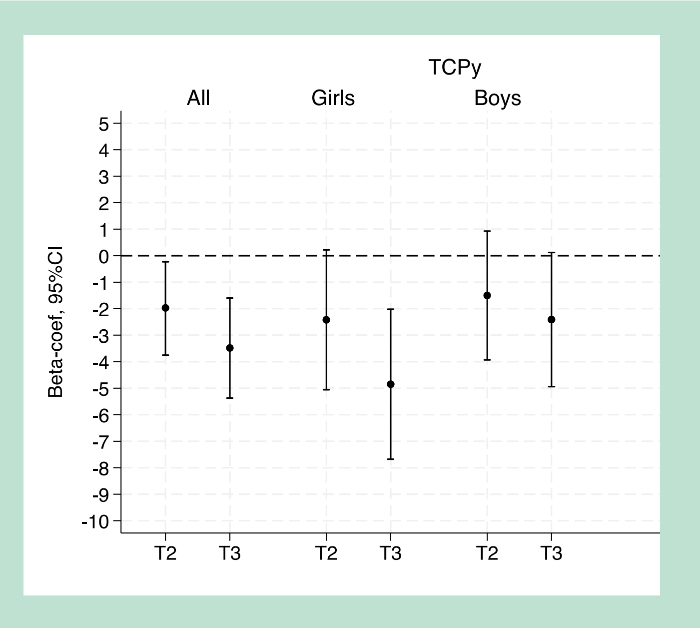

FIGURE 8. Adjusted results from linear regression analyses of associations between child TCPy (chlorpyrifos exposure) and Full-Scale Intelligence Quotient (FSIQ) at age 7 year among 1071children (460 girls and 611 boys) from the Odense Child Cohort. The Beta-coefficients present change in FSIQ scores with 95% confidence intervals (95% CI) for TCPy concentrations in the 2. (T2) or 3. (T3) tertile compared to the first tertile (T1). The models were adjusted for maternal education and for child sex (all children) and urinary creatinine concentrations.

6.2.3 Pyrethroid exposure and thyroid hormone function in pregnancy

Out of the 1183 women with available 3-PBA measurements, 817 also had available serum concentrations of thyroid hormones in first trimester of pregnancy. Of these, women taking prescribed thyroid medication (N=10) or with missing information on thyroid medication (N=10) were excluded, leaving 797 women eligible for data analyses.

Higher pre-pregnancy BMI was associated with higher 3-PBA and with higher fT3 and lower fT4 (Table 8). Further, smokers had lower TSH and higher fT3 and a tendency to higher 3-PBA. Nulliparous women had lower TSH and fT3. A total of 68 (8.5%) of the women were TPOab positive, i.e., they had thyroid peroxidase antibody concentrations above 34 kIU/L (Andersen et al., 2021b).

Table 9 presents the results from the linear regression analyses of associations between urinary 3-PBA concentrations and serum concentrations of TSH, fT4 and fT3. The concentration of fT3 increased across the tertiles of 3-PBA and women in the upper 3-PBA tertile had a significantly higher concentration than those in the first tertile. The analyses were adjusted for urinary creatinine, pre-pregnancy BMI, and smoking. Further adjustment for age and education level did not change the results.

Associations between 3-PBA and fT3 and fT4 were affected by TPOab status (Table 10). Among TPOab positive women, an inverse association between 3-PBA and fT4 was seen leading to a significant increase in the fT3/fT4 ratio across 3-PBA tertiles.

TABLE 8. Pregnancy urinary concentrations of 3-PBA and serum concentrations of thyroid hormones among
797 women from the Odense Child Cohort according to population characteristics

		Median (5th; 95th percentile)			TPOab positive
N (%)	3-PBA (ng/ml)	TSH (mIU/L)	fT4 (pmol/L)	fT3 (pmol/L)[a]	N(%)
797 (100)	0.20 (<LOD; 2.08)	1.44 (0.26; 3.43)	13.9 (11.4; 17.5)	4.65 (3.88; 5.54)	68 (8.5)
357 (44.8)	0.19 (<LOD; 1.96)	1.39 (0.24; 3.43)	14.1 (11.3; 18.2)	**4.73 (3.87; 5.74)**	27 (7.6)
291 (36.5)	0.22 (<LOD; 2.40)	1.56 (0.26; 3.55)	13.9 (11.5; 17.1)	**4.60 (3.83; 5.43)**	26 (8.9)
149 (18.7)	0.21 (0.03; 2.08)	1.44 (0.25; 3.36)	13.7 (11.1; 17.4)	**4.53 (3.84; 5.44)**	15 (10.1)
222 (27.9)	0.20 (<LOD; 2.16)	1.35 (0.21; 3.37)	14.0 (11.3; 18.4)	**4.75 (3.90; 5.87)**	18 (8.1)
408 (51.2)	0.20 (<LOD; 1.85)	1.48 (0.25; 3.74)	13.8 (11.4; 17.1)	**4.64 (3.83; 5.43)**	34 (8.3)
159 (19.9)	0.21 (0.04; 3.16)	1.46 (0.33; 3.43)	13.9 (11.6; 17.1)	**4.53 (3.84; 5.42)**	15 (9.4)
8 (1.0)					
21 (2.6)	**0.17 (0.04; 1.17)**	1.50 (0.02; 3.21)	**14.2 (11.1; 18.6)**	**4.41 (3.36; 5.71)**	3 (14.3)
487 (61.1)	**0.19 (<LOD; 1.39)**	1.51 (0.29; 3.59)	**14.0 (11.5; 17.3)**	**4.56 (3.81; 5.43)**	40 (8.2)
289 36.3)	**0.29 (<LOD; 4.03)**	1.25 (0.24; 3.26)	**13.7 (11.3; 17.7)**	**4.82 (4.06; 5.78)**	25 (8.7)
776 (97.4)	0.20 (<LOD; 2.08)	**1.45 (0.26; 3.44)**	13.9 (11.4; 17.4)	**4.64 (3.85; 5.52)**	67 (8.6)
21 (2.6)	0.36 (<LOD; 2.48)	**1.06 (0.03; 2.50)**	13.3 (11.4; 26.9)	**5.20 (3.81; 9.36)**	1 (4.8)
466 (58.5)	0.21 (<LOD; 2.15)	**1.51 (0.30; 3.74)**	13.9 (11.4; 17.7)	**4.72 (3.88; 5.59)**	36 (7.7)
331 (41.5)	0.19 (0.03; 1.87)	**1.28 (0.23; 3.17)**	13.9 (11.4; 17.1)	**4.56 (3.82; 5.46)**	32 (9.7)

[a]fT3 was available for 796 samples; M: median; p5-p95: 5th-95th percentiles; bold indicates statistically significant difference (p<0.05) between groups (Kruskal Wallis Test).

TABLE 9. Adjusted associations between urinary 3-PBA concentrations and TH-hormones in pregnancy.
□ expresses percentage change in the thyroid hormones (95% confidence interval) compared to the low 3-PBA tertile.

	TSH	fT4	fT3	fT3/fT4 ratio
3-PBA-tertile	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)
Low	Reference	Reference	Reference	Reference
Medium	-11.8 (-24.6; 3.1)	1.6 (-0.7; 4.0)	1.2 (-0.7; 3.3)	-0.3 (-2.5; 1.8)
High	-0.5 (-16.6;18.8)	1.2 (-1.4; 3.9)	**2.9 (0.7; 5.2)**	1.7 (-0.8; 4.2)
P trend	*0.91*	*0.35*	***0.01***	*0.20*

[a]Adjusted for urinary creatinine (mmol/L), pre-pregnancy BMI (continuous), and smoking (yes/no); bold indicate p<0.05.

TABLE 10. Adjusted associations between 3-PBA tertiles and fT4, fT3 and fT3/fT4-ratio stratified by TPOab-status.

	TPOab negative (n=729)			TPOab positive (n=68)		
	fT4	fT3	fT3/fT4-ratio	fT4	fT3	fT3/fT4-ratio
3-PBA-tertile	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)
Low	Reference	Reference	Reference	Reference	Reference	Reference
Medium	1.8 (-0.6; 4.3)	1.1 (-1.0; 3.3)	-0.7 (-2.9; 1.6)	-1.4 (-9.7; 7.8)	2.6 (-3.6; 9.4)	4.1 (-4.0; 12.7)
High	2.0 (-0.7; 4.8)	**2.7 (0.4; 5.2)**	0.7 (-1.9; 3.4)	-6.4 (-14.7; 2.7)	5.3 (-1.6; 12.6)	**12.4 (3.3; 22.4)**
P trend	*0.14*	***0.02***	*0.62*	*0.16*	*0.13*	***0.01***

[a]Adjusted for urinary creatinine (mmol/L), pre-pregnancy BMI (continuous), and smoking (yes/no); bold indicate p<0.05.

6.2.4 Exposure and blood pressure in childhood

For this sub-study, mother-child pair (singletons) with either urinary concentrations of insecticide metabolites from the mother in GW 28 (N=1183) or from the child at age 5 years (N=1237) and available measurement of blood pressure (BP) at age 18 months (N=769) or at 3 years (N=1396), 5 years (N=1451), or 7 years (N=1414) were included. Thus, the number of children with available exposure and BP measurements varied across the age groups. Child BP (SBP and DBP) was significantly associated with child BMI Z-score in all four age groups (Table 11). No other consistent associations were seen between population characteristics and child BP.

No associations between prenatal maternal or childhood pyrethroid exposure, estimated by urinary concentrations of 3-PBA and trans-DCCA, and BP during childhood were observed, neither in unadjusted analyses (Table 12 and Table 13) or in adjusted regression models (Table 14 and Table 15). Including maternal age, BMI, or smoking status in the regression models for prenatal exposure did not change the results. None of the associations were modified by sex (all p-values for interaction were > 0.1) and therefore, sex-stratified analyses were not performed.

TABLE 11. Blood pressure during childhood according to maternal and child characteristics

		Blood pressure 18 months			Blood pressure 3 years			Blood pressure 5 years			Blood pressure 7 years	
		Systolic	Diastolic		Systolic	Diastolic		Systolic	Diastolic		Systolic	Diastolic
Characteristics	N*	Mean (SD)	Mean (SD)	N*	Mean (SD)	Mean (SD)	N*	Mean (SD)	Mean (SD)	N*	Mean (SD)	Mean (SD)
Total	769			1396			1451			1414		
Maternal age at birth (years)												
<30	295	100.2 (10.4)	62.4 (8.9)	566	**99.0 (7.1)**	62.1 (5.8)	594	101.4 (7.3)	64.1 (5.6)	579	**105.1 (8.2)**	66.2 (5.7)
30-35	316	100.0 (8.9)	62.4 (7.5)	554	**100.1 (7.1)**	62.6 (5.7)	576	100.6 (6.8)	63.6 (5.9)	563	**103.7 (7.6)**	65.9 (5.9)
>35	158	100.8 (10.3)	63.8 (8.2)	276	**100.1 (7.4)**	62.5 (5.4)	281	101.4 (7.3)	63.7 (5.7)	272	**105.0 (7.7)**	66.6 (5.6)
Maternal pre-pregnancy BMI (kg/m^2)												
<18.5	26	101.1 (13.7)	60.6 (7.6)	37	**101.6 (8.9)**	64.2 (6.5)	40	100.3 (6.0)	63.4 (5.7)	41	104.9 (7.1)	66.9 (4.1)
18.5-25	469	100.3 (9.3)	62.6 (8.0)	874	**99.2 (7.1)**	62.1 (5.7)	916	100.8 (7.1)	63.6 (5.9)	888	104.2 (8.0)	65.9 (6.0)
>25	274	100.0 (10.1)	63.1 (8.6)	485	**100.2(7.1)**	62.8 (5.5)	495	101.5 (7.2)	64.2 (5.5)	485	105.0 (7.8)	66.6 (5.4)
Maternal parity												
1	408	100.4 (9.9)	62.8 (8.6)	731	99.4 (7.1)	62.5 (5.5)	789	**101.4 (7.2)**	64.0 (5.8)	751	104.7 (7.7)	66.3 (5.8)
>1	361	100.1 (9.6)	62.6 (7.8)	665	99.9 (7.3)	62.3 (5.8)	662	**100.6 (7.1)**	63.7 (5.7)	663	104.3 (8.2)	66.0 (5.7)
Maternal smoking												
No	732	100.4 (9.8)	62.8 (8.2)	1346	99.6 (7.2)	62.3 (5.6)	1392	101.0 (7.1)	**63.7 (7.7)**	1358	104.5 (7.9)	66.1 (5.8)
Yes	36	98.1 (8.4)	61.3 (8.5)	48	101.3 (7.7)	63.3 (6.0)	57	102.1 (8.4)	**65.7 (6.5)**	54	105.4 (7.2)	67.0 (4.8)
Maternal educational level												
Low	213	100.4 (10.1)	62.4 (8.1)	367	99.6 (7.0)	62.2 (5.5)	384	100.9 (7.3)	64.0 (5.8)	375	104.9 (7.7)	66.3 (5.6)
Intermediate	363	99.9 (9.7)	62.9 (8.4)	705	99.4 (7.2)	62.3 (5.8)	716	101.1 (7.1)	63.8 (5.7)	705	104.4 (8.0)	66.0 (5.8)
High	186	100.7 (9.6)	62.6 (8.1)	303	100.4 (7.5)	62.8 (5.7)	328	101.1 (7.1)	63.7 (5.8)	312	104.5 (8.0)	66.3 (6.0)
Child Sex												
Boy	438	100.8 (9.5)	62.9 (8.3)	751	99.9 (7.2)	62.4 (5.7)	785	101.2 (7.0)	63.7 (5.6)	771	104.3 (7.6)	**65.5 (5.7)**
Girl	331	99.6 (10.0)	62.4 (8.1)	645	99.4 (7.2)	62.4 (5.7)	666	101.0 (7.2)	64.0 (5.9)	643	104.8 (8.3)	**67.0 (5.8)**

		Blood pressure 18 months			Blood pressure 3 years			Blood pressure 5 years			Blood pressure 7 years	
Birthweight (grams)												
<3500	338	99.7 (9.2)	**62.0 (8.1)**	629	99.7 (7.1)	62.3 (5.7)	638	101.4 (7.1)	64.1 (5.5)	634	104.6 (7.7)	66.4 (5.7)
≥3500	431	100.7 (10.2)	**63.2 (8.3)**	767	99.6 (7.3)	62.5 (5.7)	813	100.8 (7.1)	63.6 (5.9)	780	104.5 (8.1)	66.0 (5.8)
Preterm birth < 37 weeks												
No	743	100.3 (9.8)	62.7 (8.2)	1340	99.6 (7.2)	62.4 (5.7)	1395	101.0 (7.2)	63.8 (5.8)	1355	104.5 (7.9)	66.2 (5.8)
Yes	23	100.4 (8.7)	62.7 (10.6)	51	101.2 (6.5)	62.8 (5.7)	52	100.8 (6.1)	63.4 (5.8)	54	104.4 (7.3)	66.4 (4.8)
Breastfeeding (months)												
< 6	260	100.2 (10.3)	63.2 (8.3)	452	100.0 (7.1)	62.7 (5.7)	467	101.3 (7.0)	64.1 (6.0)	458	104.5 (7.6)	66.3 (5.4)
≥ 6	357	100.3 (9.1)	62.8 (8.2)	642	99.7 (7.1)	62.4 (5.6)	687	100.9 (6.7)	63.7 (5.6)	661	104.5 (7.8)	66.1 (5.7)
Child BMI Z-score (SD)												
< -1	151	**97.9 (10.5)**	**60.5 (6.8)**	221	**98.4 (7.0)**	**61.5 (5.3)**	276	99.7 (6.4)	**63.6 (5.7)**	267	**103.0 (7.8)**	**65.4 (5.6)**
-1-1	518	**100.1 (9.2)**	**62.8 (8.3)**	949	**99.4 (7.1)**	**62.3 (5.7)**	977	101.0 (7.0)	**63.7 (5.7)**	909	**104.3 (7.6)**	**66.0 (5.7)**
≥ 1	83	**105.9 (9.9)**	**66.4 (9.1)**	186	**102.2 (7.4)**	**64.0 (5.5)**	173	103.0 (8.0)	**64.8 (6.0)**	207	**107.6 (8.6)**	(5.9)

*Deviation from the total numbers for some characteristic categories is due to missing information. Bold indicates statistically significant difference (p<0.05) between groups (Kruskal Wallis Test).

TABLE 12. Mean (SD) systolic (SBP) and diastolic (DBP) blood pressure (mmHg) in children between age 1.5 and 7 years according to maternal pyrethroid metabolite concentrations.

	Age group							
	1.5 years (n=515)		3 years (n=914)		5 years (n=899)		7 years (n=879)	
3-PBA tertiles	SBP	DBP	SBP	DBP	SBP	DBP	SBP	DBP
Low (<LOD – 0.125 ng/ml)	101.0 (10.1)	62.5 (8.8)	99.4 (7.2)	62.7 (6.0)	101.0 (7.3)	63.9 (5.8)	105.3 (9.3)	66.3 (6.3)
Medium (0.126-0.364 ng/ml)	100.0 (10.2)	62.6 (7.5)	100.0 (7.4)	62.8 (5.8)	101.3 (6.8)	63.6 (5.7)	104.7 (7.5)	66.2 (5.3)
High (>0.364 ng/ml)	100.5 (8.4)	63.0 (7.5)	99.7 (7.0)	62.4 (5.5)	101.1 (7.3)	64.1 (6.0)	104.4 (8.0)	66.3 (5.5)
t-DCCA								
< LOD	100.5 (9.5)	62.6 (8.0)	99.8 (7.2)	62.7 (5.6)	101.2 (7.1)	63.9 (5.8)	104.8 (8.3)	66.3 (5.8)
≥ LOD (0.4 ng/ml)	100.0 (10.0)	63.1 (7.3)	98.8 (6.7)	62.2 (6.6)	100.9 (6.9)	63.5 (5.9)	104.6 (8.3)	66.4 (5.2)

TABLE 13. Mean (SD) systolic (SBP) and diastolic (DBP) blood pressure (mmHg) in children at age 5 and 7 years according to child pyrethroid metabolite concentrations at age 5 years.

s	Mean (SD)			
Age groups	5 years (n=1202)		7 years (n=1159)	
3-PBA tertiles	SBP	DBP	SBP	DBP
Low (<LOD – 0.102 ng/ml)	100.9 (7.2)	63.6 (5.9)	104.0 (7.7)	66.1 (5.7)
Medium (0.103 – 0.281 ng/ml)	101.4 (6.7)	64.4 (5.7)	104.8 (7.4)	66.4 (5.7)
High (> 0.281 ng/ml)	101.0 (7.3)	63.5 (5.3)	104.5 (7.4)	65.8 (5.5)
trans-DCCA				
< LOD	101.2 (7.2)	63.9 (5.7)	104.4 (7.5)	66.2 (5.6)
≥ LOD (0.4 ng/ml)	100.3 (6.2)	63.4 (5.2)	104.3 (7.6)	65.7 (6.0)

TABLE 14. Adjusted associations between maternal urinary pyrethroid metabolite concentrations (ng/ml) and systolic blood pressure (SBP) and diastolic blood pressure (DBP) (mmHg) in children at age 1.5 to 7 years. expresses difference in mean SBP/DBP with 95% confidence intervals (95% CI) compared to the lowest exposure tertile (3-PBA) or below LOD (trans-DCCA).

	β (95% CI)							
Age groups	1.5 years		3 years		5 years		7 years	
3-PBA tertiles	SBP	DBP	SBP	DBP	SBP	DBP	SBP	DBP
Low	Ref		Ref		Ref		Ref	
Medium	-0.4 (-2.5; 1.7)	0.1 (-1.6; 1.8)	1.1 (-0.1;2.2)	0.3 (-06; 1.3)	0.4 (-0.8; 1.5)	-0.3 (-1.2; 0.7)	-0.4 (-1.8; 0.9)	-0.2 (-1.1; 0.7)
High	0.9 (-1.4; 3.3)	0.5 (-1.4; 2.5)	0.7 (-0.7; 2.0)	-0.3 (-1.; 0.8)	0.1 (-1.1; 1.4)	0.0 (-1.0; 1.1)	-0.9 (-2.3; 0.6)	-0.3 (-1.3; 0.8)
P trend	0.46	0.60	0.30	0.66	0.83	0.94	0.25	0.59
trans-DCCA								
< LOD	Ref		Ref		Ref		Ref	
≥ LOD	-0.1 (-2.7; 2.4)	0.1 (-2.0; 2.2)	-1.1 (-2.5; 0.4)	-0.5 (-1.7; 0.7)	-0.3 (-1.8; 1.2)	-0.7 (-1.9; 0.5)	-0.1 (-1.8; 1.6)	0.2 (-1.0; 1.4)

[a]Adjusted for child BMI z-score, child sex and maternal urinary creatinine concentration (g/L).

TABLE 15. Adjusted associations between child (5 years) urinary pyrethroid metabolite con-centra-tions (ng/ml) and child systolic blood pressure (SBP) and diastolic blood pressure (DBP) (mmHg) at age 5 and 7 years. □ expresses difference in mean SBP/DBP with 95% confi-dence intervals (95% CI) compared to the lowest exposure tertile (3-PBA) or below LOD (trans-DCCA).

	β (95% CI)			
Age group	5 years		7 years	
Child exposure	SBP	DBP	SBP	DBP
3-PBA tertiles				
Low	Ref		Ref	
Medium	0.5 (-0.5; 1.5)	0.8 (0.0; 1.6)	0.9 (-0.2; 2.0)	0.4 (-0.5; 1.2)
High	0.0 (-1.1; 1.1)	-0.1 (-1.0; 0.8)	0.3 (-0.9; 1.5)	-0.4 (-1.3; 0.5)
P trend	0.91	0.72	0.69	0.33
trans-DCCA				
< LOD	Ref		Ref	
≥ LOD	-0.8 (-2.1; 0.4)	-0.4 (-1.5; 0.6)	-0.3 (-1.6; 1.1)	-0.6 (-1.6; 0.4)

[a]Adjusted for child BMI z-score, child sex and child urinary creatinine concentration (g/L)

7. Discussion

7.1 Exposure level

In this project, urinary concentrations of pyrethroid metabolites were used as biomarkers for the internal exposure level among Danish pregnant women and 5 years old children. The metabolite, 3-PBA, reflecting the combined exposure to most pyrethroids, was detectable in 90.4 % of the pregnant women and 83.3 % of the children indicating a widespread exposure to pyrethroids in the population. However, the exposure levels, i.e., 3-PBA concentrations, were low compared to most other biomonitoring studies performed in the EU (Andersen et al., 2022b) and considerably lower than concentrations reported from studies in Asia and the US performed since 2000 (Lehmler et al., 2022).

Compared to other European studies with urine samples collected in the same period as the women in the OCC (i.e., around 2010-12), the median 3-PBA concentration of 0.21 µg/L was comparable with concentrations reported among women after delivery in Sweden (0.22 µg/L) (Gyllenhammar et al., 2017), an urban adult population in Poland (0.26 µg/L) (Wielgomas et al., 2013), and adults in Germany (0.22 µg/L) (Schettgen et al., 2016) but lower than pregnant women in France (0.36 µg/L) (Dereumeaux et al., 2018) and adults from Athens in Greece (0.50 µg/L) (Li and Kannan, 2018). In general, 3-PBA concentrations in general population groups were highest in studies with samples collected after 2015 in countries from the southern part of EU (Andersen et al., 2022b). This might indicate a higher use of pyrethroids as biocides for indoor insect control in these countries while the general population (without occupational exposure) in countries with colder climate like Denmark is assumed to be mainly exposed from residues in food. However, a few studies from the Southern part of EU, based on the French PELAGIE birth cohort (Viel et al., 2015) and the Spanish INMA-Granada cohort (Freire et al., 2021) reported very low 3-PBA concentrations, e.g., below the LOD of 0.008 µg/L in the PELAGIE cohort (Viel et al., 2017; Viel et al., 2015). The exposure levels in these studies were probably underestimated because no deconjugation step was included in the analytical method and pyrethroid metabolites are mainly excreted in urine as glucuronide conjugates (Andersen et al., 2022b; Baker et al., 2004).

In the OCC, the median 3-PBA concentration was slightly lower among the children than the mothers (0.18 vs 0.21 µg/L) from the OCC. This finding was unexpected, since children are assumed to be higher exposed from pesticide residues in food because they have a relatively higher food intake per kg body weight than adults. Accordingly, some other studies with urine samples obtained from both children and adults within the same country and time period, found higher 3-PBA concentrations in children than adults, e.g., medians of 0.29 vs 0.23 µg/L, respectively, in Poland (Wielgomas and Piskunowicz, 2013), and 0.40 vs 0.24 µg/L in Slovenia (Bravo et al., 2020). The median concentration among the OCC children was also considerably lower than among 10-16 years old children sampled in 2010-12 (0.56 µg/L) from the Danish Greenhouse Children Cohort (Andersen, 2021) although an increasing time-trend in exposure was expected as seen among young adults in Sweden (Noren et al., 2020). A possible explanation could be a shift towards a higher intake of organic food in the OCC families after the children were born. This explanation is also supported by a lower median concentration of the chlorpyrifos metabolite, TCPy, among the children than their mothers in the OCC (1.15 vs 1.65 µg/L), since lower 3-PBA and TCPy concentrations were seen in individuals who predominantly eat organic food (Baudry et al., 2019).

Anyway, the median 3-PBA concentration of 0.18 µg/L in urine samples collected in 2016-18 from the OCC children was much lower than in samples collected since 2015 among children from Cyprus (median 1.93 µg/L) (Makris et al., 2022), the Valencia region in Spain (1.63 µg/L) (Fernández et al., 2020), Belgium (0.98 µg/L) (Pirard et al., 2020), Italy (0.50 µg/L) (Bravo et al., 2019), and Slovenia (0.40 µg/L) (Bravo et al., 2020).

Assessment of individual exposure levels in this project was based on insecticide metabolite concentrations measured in a single spot urine sample. Pyrethroids and chlorpyrifos are rapidly metabolised and excreted from the body within few days and therefore urinary metabolite concentrations reflect only recent exposure. Urine concentrations of the more specific metabolites (trans- and cis-DCCA, cis-DBCA, CFCA, and 3-F-PBA) will depend on recent exposure to the specific parent compounds. This fact combined with rather high LODs (0.2-0.5 µg/L) for these metabolites in our analytic method explain the low detection frequencies for these metabolites. Since the total pyrethroid exposure over time can be assumed to be rather continuous in populations exposed mainly from residues in the diet, like the OCC, the urinary 3-PBA concentration in spot urine samples is considered to be a valid exposure biomarker. Accordingly, 3-PBA was detected with similar detection rates and geometric mean concentrations across pregnancy among women who provided repeated samples (Barkoski et al., 2018; Watkins et al., 2016). However, dietary habits will likely change during pregnancy and childhood and therefore the concentration measured in one spot urine sample will not always reflect the exposure during the most vulnerable periods of pregnancy or childhood., i.e., some exposure misclassification is likely.

To enable comparison of exposure levels between pyrethroids and chlorpyrifos in this study and with chlorpyrifos exposure in future studies, results on urinary TCPy concentrations from the OCC were included. These concentrations reflect dietary exposure from imported food items solely, since chlorpyrifos has not been approved for use in Denmark for decades and never for agricultural use. Until 2020, chlorpyrifos was one of the most widely used insecticides in the EU and worldwide (Wolejko et al., 2022) with frequent detection of residues in food items (Fødevareinstituttet, 2019). Accordingly, TCPy was detectable in almost all the urine samples and the concentrations of this specific metabolite were higher than concentrations of the common metabolite, 3-PBA, used as biomarker for the combined pyrethroid exposure, i.e., 4-8-fold higher urinary TCPy than 3-PBA concentrations expressed as µg/L and 4.5-9-fold higher expressed as mmol/L among both the mothers and children from the OCC. The TCPy concentrations were comparable or lower than in other studies from the EU (Andersen et al., 2022b).

After April 2020 chlorpyrifos (and chlorpyrifos-methyl) has been banned for use in the EU and the maximum residue limit was lowered to the default value of 0.01 mg/kg for all food items in November 2020. Thus, the exposure level has probably been reduced after the urine samples analyzed in this project were collected.

7.2 Developmental neurotoxicity

In this large prospective study of mother-child pairs from the OCC, we did not find any statistically significant associations between low-level pyrethroid exposure in pregnancy and increased risk of high scores (above the 90th percentile) on the CBCL autism (PDP/ASD) scale at age 2-4 years or the ADHD scale at age 5 years or with deficits in cognitive function at age 7 years. Further, low pyrethroid exposure in childhood at age 5 years was not significantly associated with risk of higher ADHD scores at age 5 or lower IQ-scores at age 7 years.

Thus, our previous finding from the OCC of a significant association between prenatal pyrethroid exposure and higher ADHD scores at 2-4 years-of-age (Dalsager et al., 2019) was not apparent at age 5 years, although the ORs were still above 1 for 3-PBA (Table 5). Among several possible explanations are the smaller sample size at age 5 (N= 614) than age 2-4 years (N=936) causing less statistical power and selection bias, as families of children with a high

ADHD score might be less likely to participate in the 5-year follow-up. Furthermore, more families with higher exposure, i.e., maternal 3-PBA in the upper and middle tertile, did not participate in the follow-up at age 5 compared to families in the lowest 3-PBA-tertile. Thus, the prenatal exposure level was slightly reduced in the study sample at age 5 years (median: 0.20 µg/L) compared to the study sample at age 2-4 years (0.24 µg/L). An illustration of this selection is that out of 71 children from the high 3-PBA tertile with an ADHD score $\geq$ the 90th percentile at age 2-4 years, 15 (21%) scored $\geq$ the 90th percentile also at age 5 years, 24 (34%) scored below the 90th percentile, but 32 (45%) did not participate at age 5 years. Finally, it might be speculated if adaptive brain development and/or more external stimulation mask underlying/subthreshold ADHD symptoms as the children get older.

As described in the Introduction and in a recent literature review (Andersen et al., 2022a) several other epidemiological studies have investigated potential associations between prenatal or childhood pyrethroid exposure and neurodevelopmental effects. Studies on prenatal exposure were all based on prospective birth cohorts. Among these, all the studies addressing neurobehavioral outcomes reported worse scores or higher risk of ASD/ADHD diagnosis with increasing prenatal pyrethroid exposure (An et al., 2022; Barkoski et al., 2021; Eskenazi et al., 2018; Furlong et al., 2017; Lee et al., 2022; Shelton et al., 2014; Viel et al., 2017; von Ehrenstein et al., 2019) including our previous OCC study (Dalsager et al., 2019). Impaired cognitive function, e.g., IQ, was also associated with prenatal pyrethroid exposure in some studies (Eskenazi et al., 2018; Fluegge et al., 2016; Gunier et al., 2017; Horton et al., 2011; Tanner et al., 2020; Watkins et al., 2016; Xue et al., 2013) but not in other studies (Guo et al., 2020; Hisada et al., 2017; Viel et al., 2015) including a previous study from the OCC on language development (Andersen et al., 2021a). Some of these studies were performed in populations with considerable higher urinary 3-PBA concentrations than the OCC., e.g., a cohort from South Africa with residential pyrethroid use for malaria control (geometric mean: 1.11 µg/L) (An et al., 2022; Eskenazi et al., 2018) and a cohort from South Korea (geometric mean: 0.7 µg/L) (Lee et al., 2022), but associations were also seen in some cohorts with exposure levels comparable to OCC. However, the neurodevelopmental health outcomes were evaluated by a variety of different assessment methods, and in different age groups of children, and the association estimates were reported on different scales and/or based on different exposure categories. This fact made it difficult to compare the results directly and to establish exposure-response relationship for the outcomes. Overall, the evidence for an association between prenatal pyrethroid exposure and impaired neurodevelopment in children was assessed to be strong, especially for neurobehavioral problems (Andersen et al., 2022a). Thus, the lack of significant associations between maternal 3-PBA and neurodevelopmental measures in the OCC (i.e., ADHD at age 5 years, ASD scores at age 2-4 years, and FSIQ at age 7 years) observed in this project is probably due to the low exposure level and narrow exposure contrast in this cohort and amplified by the selection bias described above.

Regarding exposure during childhood, all published studies except one (Quiros-Alcala et al., 2014) found associations with adverse neurodevelopment, especially behavioral problems such as ASD or ADHD symptoms (Hicks et al., 2017; Lee et al., 2022; Oulhote and Bouchard, 2013; Viel et al., 2017; Wagner-Schuman et al., 2015) but also cognitive deficits (van Wendel de Joode et al., 2016; Viel et al., 2015; Wang et al., 2016). One of the studies investigated associations between pyrethroid exposure and ADHD symptoms over time with exposure windows spanning from prenatal to school age and repeated examinations of the children (Lee et al., 2022). They found 3-PBA concentrations during gestation and at age 2 years to be associated with ADHD symptoms at age 6, and 3-PBA concentrations at age 4 and 6 years to be associated with ADHD symptoms at age 8 years indicating vulnerable exposure periods during both pregnancy and early childhood. The remaining studies on postnatal pyrethroid exposure were all cross-sectional without possibility to assess the temporal relationship between exposure and neurodevelopment. Although we find a reverse causation rather unlikely, it cannot be excluded that e.g., children with behavioral problems are more active and therefore eat more food with pesticide residues. However, the overall evidence suggests that also pyrethroid exposure during early childhood may also affect neurodevelopment. The lack of significant associations between 3-PBA at age 5 years and neurodevelopment in our study is most likely caused by the very low exposure level among the children. Further, 3-PBA at age 5 years may not reflect pyrethroid exposure during the most vulnerable exposure windows during early postnatal brain development i.e., 1-2 years years-of-age, but in the OCC urine samples were not collected among the children at younger age.

In contrast to the findings for the pyrethroids, childhood exposure to chlorpyrifos was significantly associated with reduced cognitive function i.e., lower FSIQ at age 7 years across tertiles of TCPy at age 5 years. For prenatal chlorpyrifos, the effect was less clear. While prenatal exposure to organophosphates, including chlorpyrifos, has been associated with cognitive deficits in several epidemiological studies, e.g., (Bouchard et al., 2011; Burke et al., 2017; Rauh et al., 2011), the impact of childhood exposure has been less investigated. In a study from the French PELAGIE cohort, lower WISC working memory scores at age 6 years were associated with the children's own urinary concentrations of nonspecific dialkylphosphate metabolites (DAP) of organophosphates but not with maternal concentrations in pregnancy (Cartier et al., 2016). Thus, brain development during early childhood may be as vulnerable as the fetal period and deserves more attention in relation to exposure to insecticides, and other neurotoxicants, in general.

7.3 Thyroid hormone disruption

As described in section 4.1.3, TH disruption is of particular concern during pregnancy, as TH plays a critical role in fetal development. Importantly, the fetus does not synthesize TH during the first months of gestation, meaning that its early development relies exclusively on maternal TH transferred through the placenta to the fetal compartment (Moog et al., 2017). Among the several mechanisms by which the TH homeostasis may be disrupted, competitive binding to TTR has been identified as a key mode of action of some pesticides, as well as of other environmental chemicals (Crivellente et al., 2019; Ouyang et al., 2017). As depicted in Figure 1, all three pyrethroids included for *in vitro* testing in this study, as well as their metabolite 3-PBA, share a common structural moiety with the TH (two phenyl rings attached via an ether bond), thus raising the hypothesis of these compounds being able to compete with T4 for TTR. In our ANSA-TTR displacement assay, none of the parent pyrethroids were shown to be able to bind to TTR. However, 3-PBA displaced ANSA from TTR in a concentration-dependent manner, with significant effects from 1.6 µM. Thus, our results highlight the potential role of metabolic activation of pyrethroids with regards to TH disruptive effects. However, the physiological relevance of the observed *in vitro* effects should be carefully analyzed. In the OCC cohort, median urinary concentrations of this metabolite in mothers and children were greatly below the *in vitro* LOEC, namely 0.21 and 0.18 µg/L (≈ 0.98 and 0.84 nM) respectively, although the maximum level detected during pregnancy in this cohort goes up to approximately 0.35 µM (75.96 µg/L) (Table 3). However, urinary 3-PBA concentrations are not directly comparable with blood concentrations of 3-PBA and very few studies have analyzed 3-PBA in blood. In a previous study on pyrethroid exposure after indoor use of pyrethroids in private Danish homes (Kilpinen et al., 2021), we found plasma 3-PBA concentrations to be above the LOD of 0.0075 µg/L in 17 out of 64 blood samples with a maximum concentration of 0.18 µg/L (≈ 0.8 nM). The maximum urine concentration of 3-PBA in that study was 7.66 µg/L and for those with detectable blood concentrations, a correlation between plasma and urine 3-PBA concentrations were found to be 0.74 (spearmans rho). In a Chinese birth cohort study including 336 pregnant women, the median concentration of 3-PBA in umbilical cord blood at delivery was 4.16 µg/L (≈ 0.02 µM) cord blood serum, the 95th percentile was 115.9 µg/L (≈ 0.54 µM), and the maximum concentration was 202.24 µg/L (≈ 0.94 µM) (Silver et al., 2016). Hence, the LOEC for 3-PBA in our study is within physiological relevant concentrations in populations with relatively high pyrethroid exposure as seen in many Asian countries (Lehmler et al., 2022). Although the 3-PBA concentrations in the OCC were considerably lower, the finding is of concern because the exposure level has been increasing in several areas.

To our knowledge, the binding of 3-PBA to TTR is a novel finding in the context of 3-PBA used as exposure biomarker for pyrethroids. However, 3-PBA was suggested as a potential anti-thyroid drug in a master thesis from 1999 (Radovanovic, 1999), because of its potent binding to TTR isolated from human plasma. This knowledge has apparently been missed and was not included in a recent EFSA toxicity assessment of pyrethroid metabolites (Hernandez-Jerez et al., 2022).

Binding of 3-PBA to TTR align with another finding from this study showing higher fT3 serum concentrations in early pregnancy across tertiles of urinary 3-PBA concentrations among the included OCC women. In accordance with our finding, a recent study, situated in a banana growing area in Costa Rica with high use of pesticides, including pyrethroids (median urinary 3-PBA of 0.69 µg/L), found a positive association between 3-PBA and fT3 among pregnant women (Corrales Vargas et al., 2022). The association was not significant, which may be due to lower sample size (n=400) and because the blood samples were collected at different time points during pregnancy, with approximately half sampled in the 2nd trimester. In contrast to these findings, a Chinese study found 3-PBA to be associated with lower fT3 among 374 women recruited in third trimester when admitted for delivery (Hu et al., 2019). A similar non-significant trend was seen for fT4. The urinary 3-PBA concentrations were higher (median of 0.48 µg/L urine) than in our study (median of 0.20 µg/L), but the smaller sample size combined with adjustment for many covariates (maternal age, pre-pregnancy BMI, education, smoking status, and the frequency of washing or peeling before vegetables and fruits intake) and sample collection in late pregnancy might explain the discrepancy in the results. Especially adjustment for the covariate “frequency of washing or peeling before vegetables and fruits intake” might have affected the observed inverse association, since this variable was stated to be associated with TH, but information on the direction as well as the likely association with 3-PBA was not provided. Finally, no association between urinary 3-PBA concentrations and serum fT4 was seen among 230 women in a Japanese birth-cohort study with samples collected between GW 10 and 12 (Zhang et al., 2013). The concentration of fT3 was not measured in that study.

In agreement with the studies from China and Japan (Hu et al., 2019; Zhang et al., 2013), we did not find any association between 3-PBA and TSH. In the study from Costa Rica, 3-PBA was reported to be associated with lower TSH (Corrales Vargas et al., 2022). The dose-response relationship was non-linear, but a significant reduction was seen at high urinary 3-PBA concentrations. However, TSH regulation is strongly affected by a pronounced rise in human chorionic gonadotropin (hCG) in early pregnancy, which stimulate T4 secretion and inhibits TSH release from the pituitary gland (Dorizzi et al., 2023). Thus, disturbance of THS in pregnancy is difficult to assess. A study including 720 pregnant women from a malaria epidemic area in South Africa, with high indoor residential application of insecticides, found maternal urinary pyrethroid metabolites, including 3-PBA (median: 1.05 µg/L) at delivery to be associated with higher TSH in neonates (Chevrier et al., 2019). In adults above 18 years-of-age from the Korean National Environmental Health Survey (n=6208), inverse associations between 3-PBA and serum concentrations of total T3 and T4, but not TSH, was observed (Hwang et al., 2019). The median urinary 3-PBA concentration was high (1.5 µg/L) compared to other studies and fT3 and fT4 were not measured. A study based on the US National Health and Nutrition Examination Survey (NHANES) in 2007-08 did not find any associations between 3-PBA and neither total nor free T3 or T4 or THS in a subsample (n=1695) from the general US population above 12 years-of-age (Jain, 2016) with a median urinary 3-PBA concentration below 0.50 µg/L (Lehmler et al., 2020).

The enzyme, thyroid peroxidase (TPO) catalyzes iodide oxidation and further incorporation in the thyroglobulin molecule and is a key enzyme in the production of T4 and T3. Thyroid autoimmunity, including elevated TPO antibodies, is a prevalent condition among pregnant women (Fernandez Martinez et al., 2018). Presence of TPO antibodies in pregnancy may inhibit TPO and cause a mild deficiency in thyroid hormone availability. In the present study, an increasing fT3/fT4 ratio was seen across 3-PBA tertiles among TPOab positive women, partly explained by a decreasing trend for fT4 among these women. Among TPOab negative women, 3-PBA was associated with higher fT3 and fT4, although only statistically significant for fT3. These results should be interpreted with caution as only 68 women were TPOab positive, but the findings might indicate that TPOab positive women are more susceptible to thyroid disturbing effects of pyrethroids because of pre-existing lower capacity to synthesize thyroid hormones. To our knowledge, no other studies have investigated potential effect modification by TPOab-status on associations between pyrethroid exposure and TH concentrations in humans.

The higher serum concentrations of fT3 associated with 3-PBA might be related to the observed capability of 3-PBA for binding to TTR and thereby compete with T3 for the binding site. In mammals T4 has a higher TTR binding affinity than T3 (Richardson et al., 2015b) and this may explain the weaker non-significant association between 3-PBA and fT4 seen among TPOab negative women. An altered ratio between T3 and T4, as observed for the TPOab positive women, might be explained by inhibition of iodothyronine deiodinase enzymes and/or TH synthesis. In rodents, the pyrethroid fenvalerate inhibited the hepatic activity of 5'-monodeiodinase, resulting in reduced concentrations of T4 in serum (Maiti et al., 1995). The pyrethroid, tetramethrin, inhibited deiodinase type 2 (DIO2), converting T4 to T3, in an *in vitro* assay based on a human recombinant DIO2 enzyme (Olker et al., 2019). In addition, some studies (Dong et al., 2019; Hallinger et al., 2017) and data from the US EPA ToxCast program found that some pyrethroids were inhibitors of the Na+/I- symporter (NIS) or TPO, indicating potential inhibition of TH synthesis. However, only few pyrethroids have been tested for TH-disruptive effects in these *in vitro* assays and none of their metabolites were included.

A clear limitation of the present study was that the urine samples used for 3-PBA analyses were collected after the blood samples used for the TH measurements. Thus, we assumed that the 3-PBA concentrations were rather stable across pregnancy as suggested in some studies (Barkoski et al., 2018; Klimowska et al., 2020; Watkins et al., 2016). Further, the effect size of the observed associations between urinary 3-PBA and fT3 and fT4 in this study was rather small and only statistically significant for fT3 and, among TPOab-positive women, also the T3/fT4 ratio. However, the urinary 3-PBA concentrations in this cohort were low compared to most other cohorts and the effects may be greater at higher exposure levels, which is of concern because of the increasing use of pyrethroids.

7.4 Cardiotoxicity

Pyrethroids are known to interfere with sodium channels, which are crucial for cardiomyocyte contraction and for neurotransmission, but oxidative stress has also been implicated as a potential cardiotoxic mechanism triggered by these insecticides (Georgiadis et al., 2018; Marques et al., 2022). Besides direct adverse effects on cardiac function, as potential teratogens, prenatal exposure to pesticides can further lead to cardiac developmental toxicity. This has been shown, for instance, for pesticides such as etridiazole, metalaxyl and methyl parathion in zebrafish models (Chen et al., 2023; Vasamsetti et al., 2020; Wu et al., 2019), permethrin in quail eggs (Curtis et al., 2021) and flusilazole and epoxiconazole in stem cell-based assays with cardiomyocyte differentiation (Lauschke et al., 2020; van Dartel et al., 2011).

In the present study, we observed effects with all three pyrethroids in our 3D hiPSCs model of cardiomyocyte differentiation, but not with the metabolite 3-PBA. The pyrethroids impaired cardiomyocyte differentiation in a concentration-dependent manner, with significant effects in the order of low micromolar levels. In terms of the human relevance of these results, an average blood concentration of 151 ng/mL (≈ 0.36 µM) cypermethrin and of 39 ng/mL (≈ 0.08 µM) deltamethrin was reported among pregnant Chinese women (Simaremare et al., 2019), while in workers from a pesticide factory in Pakistan plasma concentrations of cypermethrin were as high as 400 ng/mL (≈ 0.96 µM) (Khan et al., 2010). In umbilical cord blood from Chinese newborns maximum concentrations were 390 µg/L for cypermethrin and 502.75 µg/L (≈1.3 µM) for etophenprox, which is close to the LOEL of 1.6 µM observed for this pyrethroid in our PluriLum assay, although it might still be considered a worst-case scenario level.

We did not find any associations between prenatal or childhood pyrethroid exposure and BP in the offspring during childhood in the OCC. BP was included as the best available marker of cardiovascular function in the children. A high BP is considered the single most important modifiable risk factor for cardiovascular events in adults and childhood BP is known to predict adult BP (Magnussen and Smith, 2016). In the OCC, the BP recording procedure was standardized by measuring blood pressure twice on the left arm placed at heart level in a seated position and after a short rest using the same sphygmomanometer. In contrast to recommendations, BP was not recorded several times. Further, resting in a lying position and a longer rest period could maybe have improved the reliability of these measurements. These limitations might have reduced the sensitivity of the BP measurements. Furthermore, electrocardiograms (ECG) or heart rate viability measurements would have provided more sensitive markers of cardiac function, but such data were not available in the OCC at the time of this study.

We are not aware of other epidemiological studies investigating pyrethroid exposure and BP or other indices of cardiac function among children. In adults, urinary 3-PBA concentrations have been associated with increased risk of cardiovascular disease and coronary heart disease in studies from the US (Bao et al., 2020; Xue et al., 2021) and China (Han et al., 2017). The 3-PBA concentrations were higher than in the OCC with a geometric mean of 0.41 µg/L in the US NHANES study (Xue et al., 2021) and a median of 0.74 µg/L in the Chinese study (Han et al., 2017). No studies investigating BP in relation to pyrethroid exposure in adult populations have been identified.

8. Conclusion

The results from this project support that pyrethroids have TH disruptive and cardiotoxic properties. The generic pyrethroid metabolite, 3-PBA, was able to bind to TTR at low physiological relevant concentrations, and urinary 3-PBA concentrations were associated with higher fT3 among pregnant women in a large birth Danish cohort, the OCC, with low, mainly dietary, pyrethroid exposure. Displacement of TH from TTR in early pregnancy may disturb the transplacental transport of TH to the fetus during a very vulnerable window of development. However, in this large prospective study of mother-child pairs from the OCC, we did not find any statistically significant associations between low-level pyrethroid exposure in pregnancy and increased risk of high scores (above the 90^{th} percentile) on the CBCL autism (PDP/ASD) scale at age 2-4 years or the ADHD scale at age 5 years or with reduced cognitive function (IQ) at age 7 years. Thus, our previous finding from the OCC of a significant association between prenatal pyrethroid exposure and higher ADHD scores at 2-4 years-of-age (Dalsager et al., 2019) was no longer apparent at age 5 years, probably due to a smaller sample size and selection bias at follow-up. Furthermore, low pyrethroid exposure in childhood at age 5 years was not significantly associated with risk of higher ADHD scores at age 5 or lower IQ-scores at age 7 years.

Regarding cardiotoxicity, all three tested pyrethroids (deltamethrin, α-cypermethrin, and etofenprox), but not 3-PBA, were found to impair cardiomyocyte differentiation in a concentration-dependent manner, with significant effects in the order of low micromolar levels. These cardiotoxic effects were not mirrored in higher blood pressure related to early life pyrethroid exposure in the OCC.

Overall, the lack of significant associations between prenatal or childhood pyrethroid exposure and adverse health effects on neurodevelopment or blood pressure in the OCC, is likely attributable to a widespread but low pyrethroid exposure level in the OCC. Thus, the common pyrethroid metabolite, 3-PBA, was detectable in almost all urine samples but at rather similar low concentrations. Such a narrow exposure gradient hampers the possibility to detect an exposure related effect. Further, the 3-PBA concentrations, were low compared to most other biomonitoring studies performed in the EU and considerably lower than 3-PBA concentrations reported from studies in Asia and the US with urine samples collected within the same years, i.e., 2010-12 for the pregnant women and 2016-18 for the children. Especially 3-PBA concentrations among the children in this project were low compared to the maternal level as well as other studies and might indicate a shift towards higher intake of organic food in the families after the children were born within this rather well-educated cohort.

9. Perspectives

The results illustrate the importance of considering metabolic activation when assessing the toxicity of pesticides/chemicals and the relevance of metabolite induced effects for risk assessment. Especially regarding the TTR binding, other metabolites with structural resemblance to TH, e.g., 3-(4-hydroxyphenoxy) benzoic acid and 4-F-3PBA, should be investigated.

The obtained results from the ANSA-TTR displacement assay and the PluriBeat/PluriLum assay can be useful for substantiating Adverse Outcome Pathways (AOPs), and for further development of guidelines for cumulative risk assessment and for validating Integrated Approaches to Testing and Assessment (IATA) for regulatory purposes.

OCC is a large and well-characterized cohort, but the included families are mainly well-educated with rather high socioeconomic status (SES), and the low pyrethroid exposure level seen in this cohort might not be representative for the whole Danish population. Furthermore, exposure might have increased after the urine samples were collected in 2010/12 (mothers) and 2016/18 (children). To overcome these shortcomings, it would be highly relevant to combine data from several cohorts across different exposure levels (i.e., urinary 3-PBA concentrations) and with comparable outcome measurements (e.g., CBCL scores, WISC-IQ, blood pressure) to obtain reliable data on exposure-effect relationships, and to evaluate safe exposure levels.

Since the use of pyrethroids is assumed to be increasing, it would be very relevant to collect and analyze urine samples regularly, preferentially across age and SES groups to follow the exposure situation at population level. By including TCPy as marker of chlorpyrifos, it would also allow to follow the assumed reduction in exposure level for this substance.

Considering that several other pyrethroids as well as other environmental chemicals may act through similar mechanisms (or contribute to the same adverse outcomes through different mechanisms) as the tested pyrethroids, either in relation to cardiotoxicity/cardiac development or TH disruption, possible mixture effects arising from combined exposure to different hazardous substances at low individual levels cannot be excluded. Thus, both experimental and epidemiological studies investigating these outcomes in relation to relevant mixtures are warranted.

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Developmental exposure to pyrethroids

Pyrethroids interfere with voltage-gated sodium channels essential for nervous system and cardiac muscle function. Further, they are suggested thyroid hormones (TH) disruptors with structural resemblance to triiodothyronine (T3) and thyroxine (T4). Previously, we found pyrethroid exposure in pregnancy to be associated with ADHD symptoms at 2-4 years-of-age in the large prospective Odense Child Cohort (OCC). The objectives in the present study were to investigate if pyrethroids bind to the TH transporter protein transthyretin (TTR) and/or affect cardiomyocytes in vitro, and if maternal pregnancy THs was related to pyrethroid exposure, and whether prenatal and/or childhood exposure associate with neurodevelopment and blood pressure (BP) during childhood.

The results suggested that 3-PBA, but none of the parent compounds, bound to TTR at low concentrations (>1.6 µM). All three tested pyrethroids, but not 3-PBA, impaired cardiomyocyte differentiation in the low micromolar range. 3-PBA was detectable in most OCC-samples and associated with higher non-protein bound T3 (fT3) in early pregnancy. No associations between maternal 3-PBA and risk of autism symptoms at age 2-4 years, ADHD symptoms at 5 years, or reduced cognitive function (IQ) at 7 years were seen. Further, child 3-PBA was not associated with ADHD symptoms at 5 years or IQ-scores at 7 years. No associations between 3-PBA and BP were seen. The results support that pyrethroids have TH disruptive and cardiotoxic properties. The association between 3-PBA and fT3 among pregnant women is of concern. Lack of associations between 3-PBA and child health outcomes might be attributable to a widespread low exposure.

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