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Pesticide exposure and health risk in susceptible population groups

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Preface

The project ”Pesticide exposure and health risk in susceptible population groups” was conducted at Environmental Medicine, Institute of Public Health at University of Southern Denmark in 2014-2017 and financed by the Danish Environmental Protection Agency's Pesticide Research Programme (MST-667-00164).

The project is based on data from the Greenhouse Cohort Children (GCC) and the Odense Child Cohort (OCC). This report present results on urinary concentrations of the herbicide 2,4-D and metabolites of organophosphate and pyrethroid insecticides and investigate potential associations with selected health outcomes. Some of the results are mainly presented in two scientific papers. Results on DNA-methylation (epigenetics) in blood samples from the Greenhouse Cohort children related to maternal occupational pesticide exposure are presented in Paper 1:
“Interaction between Prenatal Pesticide Exposure and a Common Polymorphism in the PON1 Gene on DNA Methylation in Genes Associated with Cardio-metabolic Disease Risk - An Exploratory Study” published in Clinical Epigenetics 9:35 (2017) and available at: http://rdcu.be/qFoP

Results on urinary concentrations of pesticide metabolites among pregnant women from the OCC and selected health outcomes in their infants are presented in Paper 2:
“Associations of maternal exposure to organophosphate and pyrethroid insecticides and the herbicide 2,4-D with birth outcomes and anogenital distance at 3 months of age in the Odense Child Cohort” published in Reproductive Toxicology 76, 53-62 (2018) and available at:
https://doi.org/10.1016/j.reprotox.2017.12.008
The DNA methylation analyses were performed at Laboratory of Protein Chemistry, Proteomics and Epigenetic Signalling (PPES), Department of Biomedical Sciences, University of Antwerp, Belgium in collaboration with professor Greet Schoeters and professor Wim Vanden Berghe.

We are very grateful to all families participating in The Greenhouse Cohort and Odense Child Cohort. We also appreciate the skilled help from assistants and students (Hans Christian Andersen Children’s Hospital and Environmental Medicine, Institute of Public Health, University of Southern Denmark). We also want to thank The Danish Environmental Protection Agency for funding (MST-667-00164) and the reviewers for constructive comments and suggestions to this report.

Summary

Many pesticides have shown neurotoxic and/or endocrine disrupting properties in experimental studies. There is therefore a risk that pesticides may interfere with development of the brain as well as the endocrine system - especially if the exposure occurs during vulnerable time periods in foetal life or childhood. To investigate potential health effects of prenatal pesticide exposure, we have followed a cohort of children, whose mothers worked in greenhouse horticulture during pregnancy (the Greenhouse Cohort). Some of the mothers were occupationally exposed to mixtures of pesticides in the first trimester before the pregnancy was recognized and preventive measures were taken. Findings from this cohort include associations between maternal occupational pesticide exposure and impaired reproductive development in boys, earlier puberty and delayed development of the nervous system in girls, and lower birth weight followed by increased body fat accumulation during childhood. We also found that children with a certain genetic variation in the *PON1* gene were more vulnerable to pesticide-related effects. They accumulated more body fat during childhood, had higher blood pressure and enhanced serum concentration of biomarkers related to the metabolic syndrome if their mother had been exposed to pesticides during pregnancy compared to unexposed children and children without the gene variant. This finding suggests an interaction between the *PON1* gene and pesticide exposure already in foetal life, which may affect disease development later in life. A potential mechanism could be an exposure related reprogramming of metabolic pathways by altered epigenetic regulation of gene-expression in those children with the gene variant.

The associations between maternal occupational pesticide exposure in early pregnancy and a wide range of health outcomes seen in the Greenhouse Cohort children raise concern that pesticide exposures, at levels typical for the general population, could have health implications for susceptible population groups such as pregnant women and children. Besides, several population studies have reported associations between exposure to organophosphate and pyrethroid insecticides in pregnancy or childhood and child neurodevelopment. It is thus important to ensure that the pesticide exposure in the Danish population does not exceed levels that can cause adverse health effects in vulnerable populations such as children and pregnant women, including those with susceptible genotypes.

The aims of this study were therefor:

- To explore differential DNA methylation patterns between prenatally exposed children with the wild type *PON1* gene (*PON1* 192QQ genotype) and children with a variant (one or two *PON1* 192R-allels). This part of the study was based on blood samples and health outcomes from a subgroup of 48 children from the Greenhouse Cohort collected when the children were between 6 and 11 years old.

- To investigate exposure levels to pyrethroid and organophosphate insecticides in pregnant women and children recruited from the general Danish population by measuring urinary concentrations of the pesticide metabolites and to compare the concentrations with those found in other population studies. Since urine concentrations of the herbicide 2,4-D was automatically provided when analysing the pyrethroids, the results for this compound was also included. This part of the study is based on urine samples collected in 2010-12 from 858 pregnant women in the Odense Child Cohort (OCC) and from 143 children from the Greenhouse Cohort obtained at examinations in 2011-2013.

- To investigate potential associations of urinary pesticide metabolite concentrations with indicators of impaired nervous system function (motor speed function and attention) in the Greenhouse Cohort children at 11-16 years-of-age, and with birth outcomes and ano-genital distance (AGD, as marker of reproductive development) in 3 months old babies from the OCC.

We found a specific methylation profile in DNA isolated from blood samples from prenatally pesticide-exposed children with the192R-allele variant in the *PON1* gene. The methylation pattern differed from both exposed children with the QQ-genotype and from unexposed children independent of the genotype. Differentially methylated

genes were especially seen in several neuroendocrine signalling pathways related to obesity and cardiovascular diseases and suggest a link with the metabolic effects observed in these children. Furthermore, we were able to identify possible candidate genes, which mediated the associations between pesticide exposure and increased leptin level, body fat percentage, and difference in BMI Z-score between birth and school age. The findings deserve further investigation in a larger study with quantitative data on pesticide exposure. Since approximately 40% of the population has the PON1 192R-allele variant it would also be highly relevant to investigate if this genotype plays a role for pesticide related health risk at exposure levels occurring in the general population.

Regarding exposure levels, we found widespread exposure to organophosphate and pyrethroid insecticides and to the chlorophenoxy herbicide 2,4-D with detectable metabolite concentrations in urine samples from more than 90% of the women and children in this study. The exposure levels for both organophosphates and pyrethroids were similar or higher among the children in this study than in most other published studies based on the general population without special residential exposures. This is of concern since recent studies have reported associations between urinary concentrations of the generic pyrethroid metabolite, 3-PBA, in children and impaired cognitive functions and behavioural problems at exposure levels below those found among the children in this study. We did not find any associations between the children's urinary 3-PBA concentrations and motor speed function or attention but studies including test that covers different cognitive domains and neurobehavioral outcomes are warranted. We saw an association between the girls' urinary concentrations of organophosphate metabolites (both for the total alkyl phosphate metabolites of organophosphates (∑DAPs) and for the specific metabolite of chlorpyrifos (TCPY)) and hit errors in computer performance test. This finding of impaired attention among the girls may be of concern since some experimental studies have indicated that females are more susceptible to neurotoxic effects of organophosphates than boys. Furthermore, we previously found the girls in this cohort to be more affected by maternal occupational pesticide exposure during pregnancy than the boys. Like for the pyrethroids, further investigation in a larger cohort including a broader battery of tests would be highly relevant.

Among the 857 mother-child pairs included from the OCC we found no consistent statistically significant dose-related associations between maternal urinary concentrations of pesticide metabolites and birth outcomes or AGD in the offspring. However, we found a tendency towards a dose-related elongation of AGD in girls related to maternal pyrethroid exposure (i.e., 3-PBA concentration) and maternal exposure to organophosphate insecticides (∑DAP concentration). Although these associations were not statistically significant, the finding deserves to be further investigated since it may be an indication of developmental disturbance of the brain region (hypothalamus) involved in regulation of the neuroendocrine system (Gore 2010). Such a mechanism is also in accordance with our findings of disturbed development of the reproductive -, metabolic -, and nervous system related to prenatal pesticide exposure in the Greenhouse Cohort children. Furthermore, exposure to pyrethroid and organophosphate insecticides is widespread and even weak effects might add to the combined effect of environmental endocrine disruptors on reproductive development.

The increasing use of pyrethroids both in agriculture and as biocides in residential settings combined with the findings in this study of a relatively high exposure level compared to most other studies illustrate the need for exposure monitoring as well as more studies on potential health effects of pyrethroids. Some population studies indicate adverse effects on neurodevelopment and behavioural difficulties at lower exposure levels than seen in this study. As mentioned above, our findings of a potential weak disturbance of reproductive development in females may suggest a sex-dimorphic disturbance of hypothalamic function but confirmation of such a mechanism need further studies. Both pyrethroids and organophosphate insecticides are well known neurotoxicants and developmental neurotoxicity is likely to be the most sensitive effect (Bjorling-Poulsen et al. 2008; Burke et al. 2017; Grandjean and Landrigan 2014; Lee et al. 2015a, b). However, only few studies have investigated neurodevelopmental outcomes at exposure levels occurring in the general population and especially longitudinal population studies would be highly relevant. In addition, more experimental studies on potential mechanisms would be valuable. So far, developmental neurotoxicity testing has not been required for regulatory approval of pesticides or for setting of Maximal Residue Limits (MRL) in commodities. Inclusion of developmental neurotoxicity would most likely reduce the NOAEL value for most pesticides with neurotoxic properties leading to lower ADI values and MRLs and thus offer better protection of vulnerable population groups such as pregnant women and children.

However, the human nervous system is very complex and not all functions can be adequately investigated in animal studies. Besides, concurrent exposure to various pesticides with similar mode of action (e.g., more different pyrethroids) might cause an effect even if the limit values for the individual pesticides are not exceeded. Therefor population studies using validated sensitive neurodevelopmental outcomes and valid exposure information (e.g., bio-monitoring) are needed to control safe population exposure levels.

Resume

Mange pesticider har udvist neurotoksiske og/eller hormonforstyrrende egenskaber i laboratorieundersøgelser. Der er derfor risiko for, at pesticider kan forstyrre udviklingen af hjernen såvel som af det endokrine system - især hvis eksponeringen sker i sårbare perioder i fostertilstanden eller i barndommen. For at undersøge mulige helbredseffekter af prænatal pesticideksponering har vi fulgt en kohorte af børn, hvis mødre arbejdede i væksthusgartnerier under deres graviditet (Gartnerbørn kohorten). Nogle af mødrene var erhvervsmæssigt udsat for blandinger af pesticider i første trimester, før graviditeten var erkendt og der blev iværksat forebyggende foranstaltninger. Resultaterne fra denne kohorte omfatter sammenhænge mellem mødrenes erhvervsmæssige pesticideksponering og påvirkning af kønsudvikling hos drengene og tidligere pubertet og forsinket udvikling af nervesystemet hos pigerne samt lavere fødselsvægt efterfulgt af øget opbygning af kropsfedt i løbet af barndommen. Vi fandt også, at børn med en bestemt genetisk variant af *PON1*-genet var mere sårbare overfor pesticidrelaterede effekter. Disse børn akkumulerede mere kropsfedt i barndommen, havde højere blodtryk og øget serumkoncentration af biomarkører relateret til metabolisk syndrom, hvis deres moder var blevet udsat for pesticider under graviditeten sammenlignet med ueksponerede børn og børn uden denne genvariant. Dette fund tyder på en interaktion mellem *PON1* genet og pesticideksponering allerede i fosterlivet, som kan påvirke risikoen for sygdomsudvikling senere i livet. En mulig mekanisme kan være en eksponeringsrelateret omprogrammering af metaboliske signalveje på grund af en ændret regulering af gen-ekspressionen hos børn med *PON1* genvarianten.

De sammenhænge der er set mellem mødrenes erhvervsmæssige pesticideksponering i starten af graviditeten og en række sundhedseffekter hos børnene i Gartnerkohorten, giver grund til bekymring for, at de niveauer af pesticideksponering, der er typiske for den generelle befolkning, også kan tænkes at have sundhedsmæssige konsekvenser for sårbare befolkningsgrupper som gravide kvinder og børn og at risikoen kan være yderligere forøget for særligt genetisk sårbare individer. Desuden har adskillige befolkningsundersøgelser fundet sammenhænge mellem eksponering for organofosfat- og pyrethroidinsekticider under graviditet eller i barndommen og børnenes udvikling. Det er derfor vigtigt at sikre, at pesticideksponeringen i den danske befolkning ikke overstiger niveauer, der kan forårsage uønskede helbredseffekter i sårbare befolkningsgrupper som børn og gravide, herunder dem med følsomme genotyper.

Formålet med denne undersøgelse var derfor:
• At udforske mulige forskelle i DNA-methyleringsmønstre mellem prænatalt udsatte børn med vildtypen af PON1-genet (PON1 192QQ-genotype) og børn med en variant af genet (et eller to PON1 192R-alleler). Denne del af undersøgelsen er baseret på blodprøver og sundhedsudfald fra en undergruppe af 48 børn fra Gartnerkohorten indsamlet da børnene var mellem 6 og 11 år.

• At undersøge eksponeringsniveauer for pyrethroid- og organofosfatinsekticider hos gravide kvinder og børn rekrutteret fra den generelle danske befolkning ved at måle urinkoncentrationerne af pesticidmetabolitter og sammenligne koncentrationerne med dem, der findes i andre befolkningsundersøgelser. Da urinkoncentrationen af herbicidet 2,4-D automatisk blev målt sammen med pyrethroiderne, blev resultaterne for dette stof også inkluderet. Denne del af undersøgelsen er baseret på urinprøver indsamlet i 2010-2012 fra 858 gravide kvinder fra Odense Børne kohorte (OBK) og fra 143 børn fra Gartnerkohorten, da de blev undersøgt i 2011-13.

• At undersøge mulige sammenhænge mellem koncentrationer af pesticidmetabolitter i urinprøver fra børnene i Gartnerkohorten med indikatorer for nedsat funktion af nervesystemet (motorisk hastighedsfunktion og opmærksomhed) i 11-16 års alderen
samt at undersøge sammenhænge mellem koncentrationer af pesticidmetabolitter i urin fra gravide kvinder i OBK og fødselsudfald samt ano-genital afstand (AGD, som markør for reproduktiv udvikling) hos deres 3 måneder gamle babyer.

Vi fandt en specifik methyleringsprofil i DNA isoleret fra blodprøver fra prænatalt pesticideksponerede børn med 92R-allel-varianten i PON1-genet. Methyleringsmønstret var forskelligt fra både eksponerede børn med QQ-genotypen og fra ueksponerede børn uafhængigt af PON1 genotypen. Forskelle i methylering blev især set i gener involveret i neuro-endokrine signalveje relateret til fedme og kardiovaskulære sygdomme og indikerer således et link til de metaboliske forstyrrelser, der er set hos disse børn. Desuden kunne vi identificere mulige kandidatgener, involveret i mekanismen bag de observerede sammenhænge mellem pesticideksponering og forhøjet leptinniveau, kropsfedtprocent og forskel i BMI Z-score mellem fødsel og skolealder. Resultaterne bør undersøges yderligere i en større undersøgelse med kvantitative data om pesticideksponering. Da ca. 40% af befolkningen har PON1 192R-allelvarianten, vil det også være meget relevant at undersøge, om denne genotype medfører en øget sundhedsrisiko ved udsættelse for pesticider ved eksponeringsniveauer, der forekommer i den generelle befolkning.

Hvad angår eksponeringsniveauer fandt vi en udbredt eksponering for organofosfat- og pyrethroidinsekticider samt for chlorophenoxy-herbicidet 2,4-D, idet der var målbare koncentrationer af metabolitter i urinprøver fra mere end 90% af kvinderne og børnene i undersøgelsen. Eksponeringsniveauerne for både organofosfater og pyrethroider var på samme niveau eller højere blandt børnene i dette studie end i de fleste andre undersøgelser. Dette er bekymrende, da nyere undersøgelser har rapporteret om sammenhænge mellem urinkoncentrationer af den fælles pyrethroidmetabolit, 3-PBA, hos børn og nedsat kognitive funktion og adfærdsproblemer ved lavere eksponeringsniveauer end dem, der blev fundet blandt børnene i denne undersøgelse. Vi fandt ingen sammenhænge mellem børnenes urinkoncentration af 3-PBA og deres testresultater af motoriske funktion eller opmærksomhed, men dette bør undersøges nærmere med testning af forskellige kognitive områder samt adfærdsmæssige undersøgelser. Vi så en sammenhæng mellem pigernes urinkoncentration af organofosfatmetabolitter (både for de samlede alkylfosfatmetabolitter af organofosfater (ΣDAP'er) og for den specifikke metabolit af chlorpyrifos (TCPY)) og antal fejl i en computertest af koncentrationsevne. Den nedsatte opmærksomhed blandt pigerne er bekymrende, da nogle laboratorieundersøgelser også har fundet, at hunner er mere påvirkelige for neurotoksiske virkninger af organofosfater end hanner. Desuden har vi tidligere fundet, at nervesystemet hos pigerne fra Gartnerkohorten var mere påvirket af moderens erhvervsmæssige pesticideksponering under graviditeten end drengene. Ligesom for pyrethroiderne vil det være yderst relevant at undersøge denne sammenhæng yderligere med et bredere batteri af test.

Blandt de 857 moder-barn-par, der var inkluderet fra OBK, fandt vi ingen konsistente statistisk signifikante dosisrelaterede sammenhænge mellem mødrenes urinkoncentrationer af pesticidmetabolitter og fødselsudfald (fødselsvægt og – længde samt hoved- og maveomfang) eller AGD ved 3 måneder hos børnene. Vi fandt imidlertid en tendens til en dosisrelateret forlængelse af AGD hos pigerne relateret til moderens pyrethroid-eksponering (dvs. 3-PBA-koncentration) og til moderens eksponering for organofosfater (ΣDAP-koncentration). Selvom disse sammenhænge ikke var statistisk signifikante, vil det være relevant med yderligere undersøgelser, da det kan være en indikation af udviklingsforstyrrelser i den del af hjernen (hypothalamus) som er involveret i reguleringen af det neuroendokrine system (Gore 2010). En sådan mekanisme er også i overensstemmelse med resultaterne fra børnene i Gartnerkohorten, hvor prænatal pesticideksponering var relateret til ændret udvikling af reproduktions- og nervesystem og til metaboliske forstyrrelser. Endvidere er eksponering for pyrethroid- og organofosfatinsekticider udbredt, og selv små påvirkninger kan, sammen med påvirkning fra andre hormonforstyrrende stoffer, spille en rolle for udvikling af reproduktionssystemet.

Den stigende anvendelse af pyrethroider både i landbruget og som biocider i hjemmet, kombineret med resultaterne i denne undersøgelse af et relativt højt eksponeringsniveau i forhold til andre undersøgelser, illustrerer behovet for biomonitorering af eksponeringen samt flere undersøgelser af potentielle sundhedseffekter af pyrethroid-eksponering. Nogle befolkningsundersøgelser indikerer effekter på nervesystemets udvikling og adfærdsmæssige vanskeligheder ved lavere eksponeringsniveauer end dem set i denne undersøgelse. Som nævnt ovenfor kan vores fund af en potentiel svag påvirkning af pigernes kønsudvikling måske skyldes en kønsspecifik forstyrrelse af hypothalamus ' funktion, men det kræver yderligere undersøgelser at bekræfte en sådan mekanisme. Både pyrethroider og organofosfatinsekticider er velkendte neurotoksiske stoffer, og effekter på hjernens udvikling er sandsynligvis den mest følsomme effekt (Bjorling-Poulsen et al. 2008; Burke et al. 2017; Grandjean

and Landrigan 2014; Lee et al. 2015a, b). Imidlertid er der kun få undersøgelser af mulige effekter på nervesystemets udvikling ved de eksponeringsniveauer, der forekommer i den generelle befolkning, og især longitudinelle befolkningsundersøgelser vil være yderst relevante. Derudover ville flere eksperimentelle undersøgelser af potentielle mekanismer være værdifulde. Hidtil har regulatorisk testning af effekter på nervesystemet under udvikling ikke været påkrævet i forbindelse med godkendelse af pesticider eller til fastsættelse af maksimalgrænseværdier for restkoncentrationer (MRL) i fødevarer. Inddragelse af sådanne effekter vil sandsynligvis reducere NOAEL-værdien og dermed ADI-værdien for de fleste pesticider med neurotoksiske egenskaber. Dette vil føre til lavere MRL-værdier og dermed øget beskyttelse af sårbare befolkningsgrupper som gravide og børn. Det menneskelige nervesystem er imidlertid meget komplekst, og ikke alle dets funktioner kan undersøges tilstrækkeligt i dyreforsøg. Desuden kan samtidig eksponering for flere pesticider med samme virkningsmåde (f.eks. flere forskellige pyrethroider) forårsage effekt, selvom grænseværdierne for det enkelte pesticid ikke overskrides. Derfor er befolkningsundersøgelser, der anvender validerede følsomme metoder til at undersøge nervesystemets funktion og udvikling sammen med valide oplysninger om eksponeringsniveauer (f.eks. ved hjælp af bio-monitorering), nødvendige for at kontrollere at eksponeringsniveauet i befolkningen er på et sikkert niveau.

1. Introduction

A considerably part of modern pesticides has neurotoxic and/or endocrine disrupting properties (Andersen et al. 2002; Bjorling-Poulsen et al. 2008; Orton et al. 2011) and therefore the potential to disturb development of neurobehavioral, neuroendocrine, and reproductive functions (Gore 2010; Grandjean and Landrigan 2006; Jacobsen et al. 2010; Li et al. 2008; London et al. 2012). The risk is especially high if exposure occurs during vulnerable time periods in foetal life or childhood. To investigate potential health effects of prenatal pesticide exposure, we have followed a cohort of children, whose mothers worked in greenhouse horticulture during pregnancy (the Greenhouse Cohort). Some of the mothers were occupationally exposed to mixtures of pesticides in the first trimester before the pregnancy was recognized and preventive measures were taken. Findings from this cohort include associations between maternal occupational pesticide exposure and impaired reproductive development in boys (Andersen et al. 2008; Wohlfahrt-Veje et al. 2012a), earlier puberty and impaired neurobehavioral function in girls (Andersen et al. 2015; Wohlfahrt-Veje et al. 2012b), and lower birth weight followed by increased body fat accumulation during childhood (Wohlfahrt-Veje et al. 2011).

The serum enzyme paraoxonase 1 (PON1) is a high-density-lipoprotein (HDL)-associated enzyme with antioxidant function. It protects lipoproteins from oxidative modifications, and thus protects against the development of atherosclerosis (Mackness et al. 1998). It also catalyzes the hydrolysis of a wide range of substrates including the toxic oxon metabolites of several organophosphates and therefore provides some protection against chronic exposure to these pesticides (Costa et al. 2003). Several polymorphisms in the *PON1* gene have been identified and a common polymorphism in the coding sequence, a glutamine (Q)/arginine(R) substitution at position 192, affects the catalytic capacity toward pesticides (Costa et al. 2003), and also the anti-atherogenic properties (Durrington et al. 2001). Hence, an association between the R-allele and the risk of developing cardiovascular disease (CVD) has been reported (Durrington et al. 2001). The ability of *PON1* to protect against both organophosphate toxicity and atherosclerosis is also supported by experimental studies, since *PON1*-knockout mice were more sensitive to the toxic effects of chlorpyrifos, and developed atherosclerosis when fed a high-fat diet (Shih et al. 1998). Although the *PON1* Q192R genotype seems to affect both the catalytic activity and the anti-atherogenic properties, the potential interaction between this genotype and pesticide exposures on cardiovascular risk factors has not previously been investigated. To explore such an interaction, the children of female greenhouse workers were *PON1* genotyped. We found that children with the R allele in the 192 position in the *PON1* gene were particularly susceptible and the combination of this genotype and prenatal pesticide exposure was associated with increased fat accumulation from birth to school age, altered fat distribution, higher blood pressure and enhanced serum concentration of biomarkers related to the metabolic syndrome (e.g., leptin and plasminogen activator-1)(Andersen et al. 2012a; Jorgensen et al. 2015; Tinggaard et al. 2016). This strongly indicates a gene-environmental interaction already in foetal life that might cause increased disease risk later in life. We hypothesized that reprogramming of metabolic pathways dependent of both *PON1*-genotype and prenatal pesticide exposure could be caused by altered epigenetic regulation of gene-expression. Thus, one aim of this study was to explore differential DNA methylation patterns between prenatally exposed children with the *PON1* 192QQ genotype and children with one or two *PON1* 192R-allels.

The associations between maternal occupational pesticide exposure in early pregnancy and a wide range of health outcomes seen in the Greenhouse Cohort children raise concern that pesticide exposures, at levels typical for the general population, could have health implications for susceptible population groups such as pregnant women and children. Among the findings were smaller genitals and an increased risk of cryptorchidism at 3 months-of-age in sons of occupationally pesticide-exposed mothers (Andersen et al. 2008). This finding was confirmed when information on cryptorchidism diagnosis and orchiopexy (i.e., surgery applied in more severe cases) from the Nationwide Danish health registers was used for both the Greenhouse Cohort and another Danish Cohort (The Aarhus Birth Cohort) (Gabel et al. 2011). To our knowledge, no other human studies have investigated associations between modern non-persistent pesticide exposure *in utero* or childhood and later metabolic and reproductive disturbances. However, several studies have investigated neurodevelopment in children in relation

to prenatal or childhood exposures. Most longitudinal studies reporting impaired neurodevelopment in children related to prenatal exposures, mainly to organophosphate insecticides, were performed among farm worker families as the CAMACHOS cohort from California or in other US cohorts established before chlorpyrifos and diazinon were banned for indoor residential use in 2001-04 in USA (Engel et al. 2011; Engel et al. 2016; Eskenazi et al. 2014; Rauh et al. 2012). A few studies indicate that also residential use of pyrethroids during pregnancy might affect neurodevelopment in children (Llop et al. 2013; Saillenfait et al. 2015; Watkins et al. 2016). Some recent studies, based on cohorts of pregnant women recruited from the general population and without extensive indoor pesticide use, did not find indication of impaired neurodevelopment in the children associated with maternal urinary concentrations of organophosphate and/or pyrethroid insecticide metabolites (Cartier et al. 2016; Donauer et al. 2016; Viel et al. 2015). However, associations between neurobehavioral disturbances and the children's own concentrations of pyrethroid metabolites in urine samples collected at the time of examination have been reported (Oulhote and Bouchard 2013; Viel et al. 2015; Viel et al. 2017). Differences in exposure levels, exposure routes, and timing of exposure in relation to developmental processes might explain differences in the health outcomes. We therefor aimed to investigate exposure levels to pyrethroid and organophosphate insecticides in pregnant women and children recruited from the general Danish population and to investigate potential associations with adverse health effects in the new-borns and the children. Since the method applied for detecting pyrethroid metabolites also can detect the chlorophenoxy herbicide 2,4-D we decided to also include this substance as it has been reported from experimental studies to affect the thyroid gland and to interfere with thyroid-hormone transport (European Food Safety Authority 2014) and to possess neurotoxic properties (Bortolozzi et al. 2004; Rosso et al. 2000; Sturtz et al. 2008). Furthermore, it was recently classified as "possibly carcinogenic to humans" (Group 2B) by WHO (Loomis et al. 2015) although the conclusion on the risk assessment from EFSA was that 2,4-D is unlikely to have a genotoxic potential or pose a carcinogenic risk to humans (European Food Safety Authority 2014). Knowledge on the 2,4-D exposure level in the Danish population seems therefore to be relevant.

2. Methods

2.1 Study populations

2.1.1 The Greenhouse Cohort Children (GCC)

The Danish Greenhouse Cohort was established between 1996 and 2001 where pregnant women working in greenhouses were enrolled in the study. At enrolment (gestational weeks 4-10), detailed information about working conditions, pesticide use and exposure was obtained from the women and their employers and the women were categorized as occupationally high, medium, or unexposed to pesticides. Women categorized as pesticide exposed, went on paid leave or were moved to work functions with less or no pesticide exposure shortly after enrolment. Hence, exposure classification relate to the period rather early in pregnancy prior to enrolment. The children were examined first time when they were 3 months old. Of the 203 children, 168 (91 boys and 77 girls) were classified as prenatally exposed and 35 children (22 boys and 13 girls) as unexposed (controls). The first followed-up of the children was performed in 2007 to 2008 when they were between 6 and 11 years old.

Of the 177 children examined, 133 children were from the original cohort and the remaining 44 were new control children recruited through the families who already participated. Two additional follow-up examinations took place during puberty when the children were between 10 and 16 years of age (Tinggaard et al. 2016) and 166 children participated in the first and 133 children in the last of these two follow-up examinations which took place from October 2011 to January 2012 and from March to June 2013 (Figure 2.1). The children were asked to deliver a urine sample at both examinations. Due to measurements related to metabolic function (not included in this study), the children were asked to fast overnight before one of the two examinations. Examination of fasting children was scheduled to take place in the morning. This arrangement was not possible for all children/families but we obtained urine samples from 128 fasting and 143 non-fasting children during the two examinations.

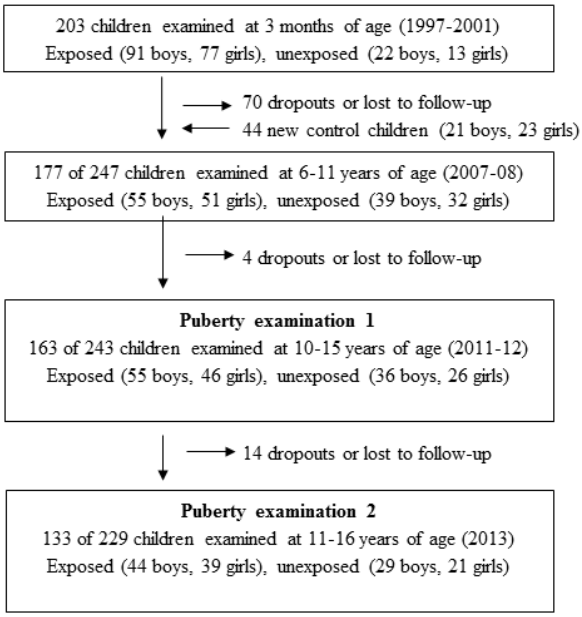

FIGURE 2.1 Flowchart of the Greenhouse Cohort children

2.1.2 Odense Child Cohort (OCC)

The Odense Child Cohort was established in collaboration between Odense Municipality, Odense University Hospital and University of Southern Denmark. All women living in the municipality of Odense and became pregnant between January 1st 2010 and December 31st 2012 were invited to participate (a total of 6,707). They were recruited either at a voluntary information meeting about ultrasound examinations, at their first antenatal visit, or at the ultrasound examination at Odense University Hospital taking place between gestational week 10 and 16 (Kyhl et al. 2015). Of the eligible women, 4,017 were informed and 2,874 (43%) agreed to participate. At the time of inclusion, the participants delivered a blood sample and filled out a comprehensive questionnaire on general health, lifestyle and social factors. In gestational week (GW) 28 (range GW 27.0 - 28.6), the mothers delivered overnight-fasting blood and urine samples and the children have been followed with thorough clinical examinations, blood/urine sampling and validated questionnaires (Kyhl et al. 2015). Samples were stored at –80°C until chemical analyses.

In this study, urine samples from 564 mothers from OCC were initially selected for pesticide analysis. Additional funding allowed analysis of 294 more samples for those pesticides measured at SDU (see below). Of these 858 urine samples, the first 200 were selected randomly and the remaining samples were selected based upon availability of information from questionnaires, birth records and AGD measurements from the three-month examinations of the children.

2.2 Pesticide analysis in urine samples

Pesticide metabolites were analysed in urine samples from the Greenhouse Cohort children and from mothers in the OCC.

Urine concentrations (µg/L) of the generic pyrethroid insecticide metabolite 3-phenoxybenzoic acid (3-BPA), three specific pyrethroid metabolites, the specific chlorpyrifos/chlorpyrifos-methyl metabolite 3,5,6-trichloro-2-pyridinol (TCPY), and the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) (Table 2.1) were measured by reversed-phase high performance liquid chromatography and tandem mass spectrometry with isotope dilution quantitation, according to the method described by Davis et al. [19] after minor modifications. Spectrophotometric determination of creatinine concentrations was conducted on a Konelab 20 Clinical Chemistry Analyzer, using a commercial kit (Thermo, Vantaa, Finland). The analysis of pyrethroid metabolites, TCPY, 2,4-D and creatinine were performed at the Environmental Medicine Laboratory, University of Southern Denmark (SDU).

Urine analyses of six unspecific organophosphate metabolites (dialkylphosphates, DAPs): diethyl phosphate (DEP), diethyl thiophosphate (DETP), diethyl dithiophosphate (DEDTP), dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP) and dimethyl dithiophosphate (DMDTP) were performed at the Flemish Institute for Technological Research NV (VITO) in Belgium using solid phase extraction (SPE) followed by Ultra Performance Liquid Chromatography-tandem mass spectrometry (UPLC-MS/MS). The urine samples were acidified and spiked with mass-labeled internal standards and concentrated using SPE. The compounds of interest were eluted with 5% NH4OH in methanol. The extract was evaporated to dryness and reconstituted with UPLC-grade water. An aliquot of the extract was injected into the LC–MS/MS system (Waters, Milford, MA, USA). The phosphate metabolites were separated on an Acquity UPLC RP shield column (100 mm × 2.1 mm; 1.7 µm). The column temperature was kept at 40 ◦C. Optimum separation was obtained with a binary mobile phase constituted of ultrapure water (solvent A) and acetonitrile (solvent B), both solvents acidified with 0.1% formic acid. The flow rate of the mobile phase was 0.4 mL/min. The UPLC system was coupled to a Waters Xevo TQ-S tandem mass spectrometer and operated in the negative electrospray ionization mode (ESI−). The system was operated in multiple reaction-monitoring (MRM) mode after selection of the characteristic precursor and product ions of each analyte. An overview of the included pesticides is shown in Table 2.1.

TABLE 2.1 Urinary pesticide metabolites measured in the study

Metabolite	Abbreviation	LOD ng/ml	Pesticide	Pesticide group
3-phenoxybenzoic acid	3-PBA	0,03	Unspecific metabolite of e.g., cypermethrin, deltamethrin, permethrin, lambda-cyhalothrin, d-phenothrin, fluvalinate-tau, esfenvalerate, fenpropathrin, flucythrinat, tralomethrin	pyrethroid
cis-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid	Cis-DCCA	0,5	cis-permethrin, cis-cypermethrin, cyfluthrin	pyrethroid
trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid	Trans-DCCA	0,4	trans-permethrin, trans-cypermethrin, cyfluthrin	pyrethroid
cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid	Cis-DBCA	0,5	deltamethrin	pyrethroid
3,5,6-trichloro-2-pyridinol	TCPY	0,2	chlorpyrifos and chlorpyrifos-methyl	organophosphate
2,4-dichlorophenoxyacetic acid	2,4-D	0,03	2,4-D	chlorophenoxy herbicide
Dimethyl phosphates: dimethyl phosphate dimethyl thiophosphate dimethyl dithiophosphate Molar sum of the three dimethyl phosphates	 DMP DMTP DMDTP ΣDMP	 0.3 0.3 0.3	Unspecific metabolite of e.g., chlorpyrifos-methyl, azinphos-methyl, dichlorvos, dimethoate, fenxhlorphos, fenthion, malathion, mevinphos, methyl parathion, phosmet, pirimiphos-metyltetrachlorviphos	organophosphate
Diethyl phosphates: diethyl phosphate diethyl thiophosphate diethyl dithiophosphate Molar sum of the three diethyl phosphates	 DEP DETP DEDTP ΣDEP	 0.3 0.3 0.3	Unspecific metabolites of e.g., chlorpyrifos, chlorethoxyphos, diazinon, ethion, parathion, phosalone, tebufos, tribufos	organophosphate
Total molar sum of dialkyl phosphates (DAPs): sum of ΣDMP and ΣDEP	ΣDAP			

2.3 Data collection from the Greenhouse Cohort children

2.3.1 DNA methylation

DNA methylation was investigated in 48 blood samples obtained from the Greenhouse Cohort children at the first follow-up examination in 2007-08. The samples were selected among pre-pubertal (Tanner Stage 1) children, whose mothers reported not to have smoked during pregnancy, and they were equally distributed between the *PON1* 192QQ and QR/RR genotype. For each genotype, we then used individual matching to select one exposed child of same sex and age for each of the controls. For the QQ-genotype, two exposed children were selected for each of two controls to obtain 24 children. Thus, in total we used data from 13 exposed and 11 unexposed children with the QQ genotype, and 12 exposed and 12 unexposed children with the QR/RR genotype (see paper 1).

Genome-wide methylation of DNA was determined by the Infinium HumanMethylation 450 BeadChip array (Illumina, San Diego, CA, USA) at Laboratory of Protein Chemistry, Proteomics and Epigenetic Signalling (PPES), Department of Biomedical Sciences, University of Antwerp, Belgium. The method is described in detail in paper 1.

2.3.2 Examination of manual motor speed and attention

2.3.2.1 Manual motor speed

In the Finger Tapping test, the child tapped a key for a series of 10-sec sessions, first completing one session with the preferred hand and one with the non-preferred hand for practice, then alternately two sessions with the preferred and non-preferred hand. We used the standard board for this test (WW-1597-NP; Psychological Assessment Resources, Odessa, FL, USA), but the thickness of the board was increased by 1 cm to allow children with small hands to move the tapping arm effortlessly (Lezak 1995). Means of the number of taps from the non-practice trials for the preferred hand, non-preferred hand, and both hands were used for data analysis.

2.3.2.2 Attention and impulsivity

Conners' Continuous Performance Test II (CPT II, version 5 for windows; Multi-Health Systems Inc., North Tonawanda, NY, USA) is a computer test that measures sustained attention and impulsivity. The child was required to press the spacebar (hits) each time a letter appeared, unless the letter was an X, during a15-min test duration. Scores derived from the test were the total number of missed responses (omissions), false positives (commissions), and the overall average reaction time (hit rc).

2.4 Data collection from the Odense Child Cohort

2.4.1 Birth outcomes and covariates

Information about birth weight (g), head circumference (cm), abdominal circumference (cm) and gestational age (days) as well as information about parity, maternal smoking, maternal pre-pregnancy BMI was obtained from birth records. Maternal educational level and ethnicity were obtained from the questionnaire filled out during pregnancy.

2.4.2 Ano-genital distance (AGD) at three months of age

A clinical examination of the children including measurements of length, weight and AGD was scheduled to take place three months after expected delivery date, regardless of actual gestational age at birth. AGD was measured using a Vernier caliper. To minimize the risk of inter-observer variations, four expert-trained technicians performed all measurements. In boys, a short AGD was measured from the center of anus to the posterior base of scrotum (AGDas) and a long AGD was measured from the center of anus to the cephalad insertion of the penis (AGDap). Correspondingly in girls, a short AGD was measured from the center of anus to the posterior fourchette (AGDaf) and a long AGD from the center of anus to the top of clitoris (AGDac) (Figure 2.2). The measurements were conducted three times for each child in order to decrease the risk of measurement bias and arithmetic mean was calculated. For all AGD measurements the coefficient of variation (CV) was below 10 %, except for AGDaf, where two girls had CV's of 10 % or 14 %, respectively (Lassen et al. 2016).

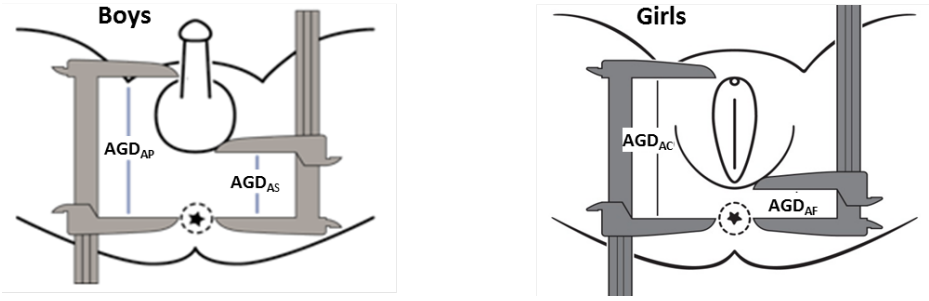

FIGURE 2.2 Illustration of AGD measurements in boys and girls

2.5 Ethics

The study was conducted in accordance with the Helsinki Declaration II with written informed consent by all mothers and/or fathers as approved by the Regional Scientific Ethical Committees for Southern Denmark (S-20070068 and S-20090130) and the Danish Data Protection Agency (j.no:1996-1200-154, 2007-41-0956, 2008-58-0035).

2.6 Data Analysis

2.6.1 DNA methylation

Differential DNA methylation was analysed according to *PON1* genotype and prenatal pesticide exposure both at the single CpG site level and at the region level. Position and regions that were significantly differentially methylated in exposed QR/RR children compared to exposed QQ and unexposed children were identified as sig-DMPs and sig-DMRs, respectively. Mediation analyses were performed to investigate whether methylation was a mediator between exposure and metabolic health outcomes in the children (body fat accumulation, body fat content, serum leptin concentration). Overlap between sig-DMPs and sig-DMRs were used as input for canonical pathway analysis and the DisGeNet platform was used to screen for gene disease associations. The data analyses are described in detail in paper 1.

2.6.2 Urinary pesticide metabolite concentrations

For all urinary pesticide concentrations, values below the limit of detection (LOD) were substituted by the specific LOD dived by the square root of two (LOD/√2). To account for urinary dilution the volume based concentrations were dived by urinary creatinine concentrations.

The DAP metabolite concentrations were converted from µg per litre urine to their molar concentrations (nmole per litre) and summed to obtain the total concentration of dimethyl phosphate metabolites ΣDMP (sum of DMP, DMTP, and DMDTP), diethyl phosphate metabolites ΣDEP (sum of DEP, DEDTP, and DEDTP), and total ΣDAP (ΣDEP plus ΣDMP).

Geometric mean of the urinary pesticide concentrations was calculated if the number of samples above the limit of detection (LOD) was higher than 20%. Median, 5th and 95th percentiles were calculated for all urinary pesticide metabolite concentrations. The values were reported as both creatinine adjusted and unadjusted concentrations.

2.6.3 Associations between urinary pesticide concentrations and health outcomes

The distributions of all urinary pesticide concentrations were skewed and therefor reported as medians. For further analyses, the concentrations were either logarithmically transformed or divided into categorical variables (tertiles or below vs above median) to minimize within-subject day-to-day variability (Meeker et al. 2005).

Differences in concentrations according to population characteristics were tested by the Mann-Whitney U test or Willcoxon rank sum test (two categories) or the Kruskal Wallis (more than two categories) tests.

Associations between urinary pesticide metabolite concentrations and health outcomes were analysed by multiple linear regression models with adjustment for potential confounders. Confounders included in the final models were factors known a priori to be important predictors of both pesticide exposure and the outcome of interest.

3. Results

3.1 Prenatal Pesticide Exposure, *PON1* R192Q genotype and related changes in DNA Methylation

The results from this sub-study are presented in paper 1.

Briefly, we found a specific methylation profile in DNA isolated from blood samples from prenatally pesticide-exposed children carrying the PON1 192R-allele. The methylation pattern differed from both exposed children with the QQ-genotype and from unexposed children with the QR/RR or QQ genotype. Differentially methylated genes were especially seen in several neuroendocrine signalling pathways including dopamine-DARPP32 feedback (appetite, reward pathways), corticotrophin releasing hormone signalling, nNOS, neuregulin signalling, mTOR signalling and type II diabetes mellitus signalling. This might suggest a link with the metabolic effects observed in these children. Furthermore, we were able to identify possible candidate genes, which mediated the associations between pesticide exposure and increased leptin level, body fat percentage, and difference in BMI Z-score between birth and school age.

3.2 Urinary pesticide concentrations in pregnant women and children from the Danish population

Table 3.1 shows urinary concentrations (unadjusted and creatinine adjusted) of 2,4-D and metabolites of organophosphate and pyrethroid insecticides among pregnant women from the OCC. One creatinine determination was lost leaving 857 creatinine-adjusted values. The unspecific organophosphate metabolites were only measured in the first 564 urine samples. The chlorophenoxy herbicide, 2,4-D, the generic pyrethroid metabolite, 3-PBA, and the specific chlorpyrifos/chlorpyrifos-methyl metabolite, TCPY, were detectable in more than 90 % of the samples while the unspecific organophosphate metabolite DEP was detectable in 81.6 % and DMP and DMTP in approximately half of the samples. The highest creatinine adjusted median concentrations were seen for DEP (2.23 μg/g crea) followed by TCPY (1.84 μg/g crea) and DMP (1.71 μg/g crea). Due to the low detection frequency of the specific pyrethroid metabolites (cis-DCAA, trans-DCCA, and cis-DBCA) these metabolites were not included in further data analysis.

Table 3.2 shows the molar concentrations of the dialkyl phosphate metabolites and TCPY. The concentration of dimethyl phosphates (ΣDMP) was a little higher than the diethyl phosphates (ΣDEP) concentration. Chlorpyrifos and chlorpyrifos-methyl are both metabolized to TCPY and while chlorpyrifos is also metabolized to diethyl phosphates (Smith et al. 2011), chlorpyrifos-methyl is metabolized to dimethyl phosphates (Aprea et al. 1997). These two organophosphates seem to account for approximately 16 % of the total organophosphate exposure estimated by the ΣDAP concentration (median TCPY/median ΣDAP = 0.16).

TABLE 3.1: Urinary concentrations (unadjusted and creatinine adjusted) of organophosphate and pyrethroid insecticide metabolites and 2,4-D in spot urine samples from pregnant women collected in gestational week 28 after over-night fasting

Pesticide group	Metabolite	N	LOD	%>LOD	Geometric mean (95% CI)	Median	5th percentile	95th percentile
	Concentration (ng/mL)							
Organophosphate insecticides	TCPY	858	0.3	93.2	1.67 (1.56, 1.78)	1.74	< LOD	8.15
	DMP	564	1.0	56.4	1.65 (1.52, 1.79)	1.24	0.71	9.92
	DMTP	564	0.3	54.8	1.00 (0.88, 1.13)	1.14	< LOD	14.7
	DMDTP	564	0.3	3.9	NC	< LOD	< LOD	< LOD
	DEP	564	0.3	81.6	1.79 (1.62, 1.98)	2.14	< LOD	9.91
	DETP	564	0.3	41.3	0.65 (0.58, 0.74)	0.21	< LOD	10.11
	DEDTP	564	0.3	0.2	NC	< LOD	< LOD	< LOD
Pyrethroid insecticides	3-PBA	858	0.03	94.3	0.22 (0.20, 0.24)	0.20	0.02	2.18
	Cis-DCCA	858	0.5	2.9	NC	< LOD	< LOD	< LOD
	Trans-DCCA	858	0.4	11.7	NC	< LOD	< LOD	1.44
	Cis-DBCA	858	0.5	2.9	NC	< LOD	< LOD	< LOD
Chlorophenoxy herbicide	2,4-D	858	0.03	97.6	0.16 (0.22, 0.27)	0.16	0.04	0.70
	Creatinine adjusted concentration (ug/g creatinine)							
Organophosphate	TCPY	857			1.90 (1.79, 2.01)	1.84	< LOD	8.27
	DMP	564			1.84 (1.69, 2.00)	1.71	0.42	10.06
	DMTP	564			1.11 (0.98, 1.26)	1.14	< LOD	15.85
	DMDTP	564			NC	< LOD	< LOD	< LOD
	DEP	564			2.00 (1.83, 2.18)	2.23	< LOD	8.47
	DETP	564			0.73 (0.65, 0.81)	0.57	< LOD	8.08
	DEDTP	564			NC	< LOD	< LOD	< LOD
Pyrethroid insecticides	3-PBA	857			0.25 (023, 0.27)	0.23	0.04	1.73
	Cis-DCCA	857			NC	< LOD	< LOD	< LOD
	Trans-DCCA	857			NC	< LOD	< LOD	1.81

	Cis-DBCA	857	NC	< LOD	< LOD	< LOD
Chlorophenoxy herbicide	2,4-D	857	0.18 (0.17, 0.19)	0.18	0.05	0.73

NC: not calculated

TABLE 3.2. Urinary molar concentrations of summed dialkyl phosphate metabolites (DAPs) of organophosphate insecticides and TCPY in spot urine samples from pregnant women collected in gestational week 28 after overnight fasting

Metabolite	N	Geometric mean (95% CI)	Median	5th percentile	95th percentile
		Concentration (nmol/L)			
ΣDMP[b]	564	26.9 (24.8, 29.3)	25.6	8.5	168.1
ΣDEP[a]	564	20.6 (18.9, 22.5)	20.0	3.8	108.5
ΣDAP[c]	564	58.7 (50.8, 59.0)	56.5	12.2	252.8
TCPY	858	8.40 (7.9, 9.0)	8.75	1.07	41.05
		Concentration (nmol/g creatinine)			
ΣDMP	564	30.0 (27.6, 32.7)	29.7	5.8	173.2
ΣDEP	564	23.0 (21.3, 24.7)	23.3	4.2	95.3
ΣDAP	564	61.0 (57.0, 65.3)	58.4	16.6	259.1
TCPY	857	9.56 (9.02, 10.13)	9.29	2.55	41.65

[a]: sum of DEP, DETP, DEDTP, [b]: sum of DMP, DMTP, DMDTP, [c]: sum of ΣDEP and ΣDMP

Table 3.3 shows the urinary pesticide metabolite concentrations in spot urine samples obtained either after overnight fasting or without previous fasting for children in the Greenhouse Cohort at age 10-16 years. The unspecific organophosphate metabolites were only determined in the non-fasting samples. Like for the pregnant women, 2,4-D, TCPY and 3-PBA were detectable in more than 90% of the samples and also DEP was found in more than 90% of the samples from the children. The highest urinary concentrations were seen for DEP, DMP and DMTP, and TCPY. The 3-PBA concentration in fasted samples was weakly but significantly correlated with the concentration in non-fasting samples (spearman's rho = 0.29, p = 0.003). For TCPY and 2,4-D no correlations between fasting and non-fasting samples were seen. The mean urine concentration of 2,4-D was approximately 30% lower in samples collected after overnight fasting. The low detection limit for this compound allowed detection in almost all the children, also after fasting. The mean urine concentration of 3-PBA was reduced by 20% in fasting samples while the mean concentration of TCPY was 17% higher in samples obtained after overnight fasting compared to non-fasting samples. None of these differences were significant at the 0.05 level (Wilcoxon Signed Ranks Test).

TABLE 3.3 Urinary concentrations (unadjusted and creatinine adjusted) of organophosphate and pyrethroid insecticide metabolites and 2,4-D in spot urine samples from children (10-16 years old) from the greenhouse cohort.

		Overnight-fasting samples (N=128)					Non-fasting samples (N=143)				
	LOD	%>LOD	GM (95% CI)	Median	5th pct	95th pct	%>LOD	GM (95% CI)	Median	5th pct	95th pct
Organophosphate insecticides					Concentration (ng/ml)						
TCPY	0.3	94.5	1.52 (1.30; 1.79)	1.55	<LOD	7.31	95.8	1.42 (1.22; 1.66)	1.43	0.22	6.05
DMP	1.0	NI	NI	NI	NI	NI	54.6	2.96 (2.42; 3.61)	1.86	1.05	25.33
DMTP	0.3	NI	NI	NI	NI	NI	69.0	2.03 (1.52; 2.71)	3.35	<LOD	23.10
DMDTP	0.3	NI	NI	NI	NI	NI	3.5	NC	<LOD	<LOD	<LOD
DEP	0.3	NI	NI	NI	NI	NI	93.7	3.25 (2.70; 3.94)	2.99	<LOD	21.46
DETP	0.3	NI	NI	NI	NI	NI	33.8	0.50 (0.40; 0.62)	<LOD	<LOD	5.66
DEDTP	0.3	NI	NI	NI	NI	NI	0	NC	<LOD	<LOD	<LOD
Pyrethroid insecticides											
3-PBA	0.03	100	0.51 (0.40; 0.65)	0.49	0.05	8.98	100	0.66 (0.53; 0.82)	0.56	0.10	8.90
Cis-DCCA	0.5	4.7	NC	<LOD	<LOD	0.44	2.8	NC	<LOD	<LOD	<LOD
Trans-DCCA	0.4	14.8	NC	<LOD	<LOD	3.06	9.8	NC	<LOD	<LOD	2.30
Cis-DBCA	0.5	0.0	NC	<LOD	<LOD	<LOD	2.1	NC	<LOD	<LOD	<LOD
Chlorophenoxy herbicide											
2,4-D	0.03	99.2	0.18 (0.16; 0.21)	0.19	0.05	0.77	100	0.26 (0.23; 0.30)	0.25	0.06	1.04
Organophosphate insecticides					Concentration (μg/g creatinine)						
TCPY			1.55 (1.36; 1.76)	1.32	<LOD	6.10		1.32 (1.12; 1.56)	1.29	0.27	7.61
DMP			NI	NI	NI	NI		2.74 (2.23; 3.38)	2.01	0.56	29.09
DMTP			NI	NI	NI	NI		1.88 (1.42; 2.50)	2.64	<LOD	25.56
DMDTP			NI	NI	NI	NI		NC	<LOD	<LOD	<LOD
DEP			NI	NI	NI	NI		3.02 (2.54; 3.61)	2.70	<LOD	18.70
DETP			NI	NI	NI	NI		0.46 (0.37; 0.58)	<LOD	<LOD	5.88
DEDTP			NI	NI	NI	NI		NC	<LOD	<LOD	<LOD
Pyrethroid insecticides											

3-PBA	0.52 (0.42; 0.65)	0.43	0.08	6.45	0.61 (0.49; 0.76)	0.46	0.11	7.67
Cis-DCCA	NC	<LOD		2.29	NC	<LOD	<LOD	<LOD
Trans-DCCA	NC	<LOD		2.82	NC	<LOD	<LOD	2.43
Cis-DBCA	NC	<LOD		<LOD	NC	<LOD	<LOD	<LOD
Chlorophenoxy herbicide								
2,4-D	0.18 (0.16; 0.21)	0.18	0.05	0.82	0.24 (0.21; 0.28)	0.25	0.06	1.12

NI: not investigated; NC: not calculat

The unspecific organophosphate metabolites were only analysed in samples obtained from non-fasting children. The molar concentrations are shown in Table 3.4. As for the pregnant women, ΣDMP was higher than ΣDEP and the molar ratio of TCPY and ΣDAP was lower (median TCPY/median ΣDAP = 0.08) indicating a lower contribution of chlorpyrifos and chlorpyrifos-methyl to the total organophosphate exposure among the children than for the OCC women.

TABLE 3.4 Urinary molar concentrations of summed dialkyl phosphate metabolites (DAPs) of organophosphate insecticides and TCPY in spot urine samples (non-fasting) from the greenhouse cohort children

Metabolite	N	Geometric mean (95% CI)	Median	5th percentile	95th percentile
Concentration (nmol/L)					
ΣDMP[b]	141	52.0 (42.9; 63.0)	53.6	11.2	385.8
ΣDEP[a]	141	27.8 (23.5; 32.9)	24.1	3.8	166.4
ΣDAP[c]	141	89.7 (75.7; 106.3)	85.6	15.5	506.0
TCPY	143	7.2 (6.1; 8.3)	7.2	1.1	30.5
Concentration (nmol/g creatinine)					
ΣDMP	140	48.1 (39.6; 58.4)	43.2	8.3	480.8
ΣDEP	140	25.9 (22.0; 30.4)	22.6	6.4	167.8
ΣDAP	140	83.2 (70.4; 98.3)	77.0	16.3	542.0
TCPY	140	6.7 (5.6; 7.9)	6.5	1.4	38.3

[a]sum of DEP, DETP, DEDTP, [b]sum of DMP, DMTP, DMDTP, [c]sum of ΣDEP and ΣDMP

The median concentrations of 3-PBA in the fasting samples from the children was approximately 2-fold higher than the median concentration from the pregnant women (also obtained after over-night fasting) whereas the concentrations of TCPY and 2,4-D were similar in the two groups (Table 3.1 and 3.3). For both population groups, significant correlations were seen for all the pesticide metabolites (Table 3.5) although the metabolites from different groups of pesticides were not strongly correlated as most of the correlation coefficients were below 0.4.

TABLE 3.5 Correlations (Spearman´s rho) between urinary concentrations of pesticide metabolites in pregnant women from OCC and children from the Greenhouse Cohort

	OCC Pregnant women					
	3-PBA	2,4-D	TCPY	ΣDMP	ΣDEP	ΣDAP
3-PBA		0.343**	0.455**	0.204**	0.346**	0.300**
2,4-D	0.315**		0.398**	0.222**	0.323**	0.289**
TCPY	0.287**	0.247**		0.380**	0.465**	0.489**
ΣDMP	0.252**	0.201*	0.343**		0.391**	0.822**
ΣDEP	0.388**	0.182*	0.312**	0.542**		0.788**
ΣDAP	0.329**	0.234**	0.390**	0.922**	0.758**	
	Greenhouse Cohort children (non-fasting samples)					

Table 3.6 and table 3.7 show urinary creatinine adjusted concentrations of pesticide metabolites in relation to population characteristics of the women from the OCC. For 23 women information on characteristics was missing because they did not answer the first questionnaire. The mean age of the remaining 834 women was 30.3 (± 4.4) years and the mean BMI was 24.7 (± 4.6) kg/m2. The majority of the women were in the intermediate educational group (51.9 %), nulliparous (55.8 %) and of European origin (97.4 %) whereas only a small proportion reported to have smoked during pregnancy (3.5 %). High educated women tended to have higher urinary concentrations of organophosphate metabolites and 2,4-D than women with lower education level although only statistically significant for TCPY and 2,4-D. A tendency towards an age-related increase in TCPY concentrations was seen but the unspecific organophosphate metabolites, DAPs, were unrelated to age. Women with low BMI had higher concentrations of 2,4-D and organophosphate metabolites than those with higher BMI whereas 3-PBA tended to increase with higher BMI. Furthermore, nulliparous women had higher concentrations of 3-PBA than multiparous women while none of the other pesticide metabolite concentrations were affected by parity.

TABLE 3.6 Urinary median concentrations (5; 95 percentiles) of TCPY, 3-PBA, and 2,4-D (μg/g creatinine) in pregnant women from OCC according to population characteristics

Characteristics	N	TCPY	3-PBA	2,4-D
Age (years)				
<25	75	1.64 (0.37; 6.34)	0.22 (0.04; 3.90)	0.16 (0.07; 0.74)
25-29	285	1.88 (0.39; 7.67)	0.24 (0.04; 1.28)	0.16 (0.05; 0.72)
30-34	325	1.80 (0.53; 8.00)	0.23 (0.05; 1.83)	0.19 (0.05; 0.66)
>34	149	1.90 (0.57; 9.34)	0.21 (0.03; 2.54)	0.18 (0.05; 0.83)
p-value		0.14	0.45	0.20
Parity				
Nulliparous	465	1.86 (0.52; 7.49)	0.25 (0.05; 1.53)	0.17 (0.05; 0.72)
Multiparous	368	1.82 (0.44; 9.05)	0.20 (0.03; 1.87)	0.18 (0.05; 0.72)
p-value		0.47	0.004	0.75
Pre-pregnancy BMI (kg/m^2)				
<18.5	21	2.19 (0.49; 9.79)	0.18 (0.07; 4.31)	0.17 (0.04; 0.92)
18.5-24-9	495	1.88 (0.50; 8.42)	0.21 (0.04; 1.32)	0.19 (0.06; 0.82)
25-29.9	215	1.73 (0.51; 6.15)	0.23 (0.06; 2.39)	0.16 (0.05; 0.58)
>29.9	103	1.79 (0.52; 7.65)	0.27 (0.02; 3.84)	0.13 (0.04; 0.40)
p-value		0.15	0.02	0.0001
Smoking in pregnancy				
No	801	1.84 (0.52; 8.24)	0.23 (0.05; 1.67)	0.18 (0.05; 0.72)
Yes	28	1.60 (0.12; 15.59)	0.25 (0.01; 3.01)	0.13 (0.01; 0.70)
p-value		0.71	0.68	0.05
Education level[a]				
Low	238	1.64 (0.38; 8.62)	0.23 (0.04; 1.54)	0.16 (0.04; 0.60)
Intermediate	432	1.89 (0.59; 7.61)	0.23 (0.05; 1.52)	0.18 (0.06; 0.75)
High	161	2.05 (0.41; 9.63)	0.22 (0.04; 3.57)	0.19 (0.05; 0.91)
p-value		0.004	0.69	0.02

[a]Low: High school and vocational education or less; Intermediate: High school + 1-3 years; High: High school + 4 years or more.

TABEL 3.7 Urinary median concentrations (5; 95 percentiles) of DAPs (nmol/g creatinine) in pregnant women from OCC according to population characteristics

Characteristics	N	ΣDMP	ΣDEP	ΣDAP
Age (years)				
<25	47	28.6 (6.7; 288.1)	23,2 (6.3; 131.6)	61.3 (18.4; 305.0)
25-29	191	30.1 (5.0; 170.6)	21.9 (3.8; 99.1)	57.7 (12.6; 259.6)
30-34	208	28.9 (6.8; 172.0)	23.1 (5.9; 86.5)	54.3 (18.2; 254.4)
>34	108	30.0 (7.0; 177.9)	25.4 (3.6; 106.0)	62.2 (15.8; 298.1)
p-value		0.87	0.57	0.73
Parity				
Nulliparous	298	28.9 (6.0; 171.0)	22.6 (5.1; 95.5)	57.6 (16.1; 260.8)
Multiparous	255	29.0 (5.8; 181.3)	25.0 (3.7; 100.9)	59.3 (16.7; 257.3)
p-value		0.94	0.52	0.70
Pre-pregnancy BMI (kg/m^2)				
<18.5	15	35.5 (8.1; 154.8)	31.1 (12.5; 94.6)	60.8 (36.0; 191.7)
18.5-24-9	333	31.6 (6.3; 173.3)	25.0 (5.2; 98.9)	61.3 (18.0; 266.0)
25-29.9	142	26.0; 5.9; 187.8)	20.4 (3.3; 83.3)	57.6 (14.3; 251.0)
>29.9	64	21.4 (4.9; 143.2)	17.6 (4.6; 145.3)	45.6 (12.5; 296.2)
p-value		0.06	0.009	0.007
Smoking in pregnancy				
No	530	30.0 (5.9; 176.1)	23.2 (5.0; 96.7)	58.9 (16.7; 259.7)
Yes	19	18.0 (5.8; 104.2)	20.5 (3.3; 58.1)	43.1 (10.8; 155.0)
p-value		0.07	0.18	0.08
Low	169	27.8 (5.2; 195.6)	23.2 (4.0; 99.6)	55.2 (12.9; 269.6)
Intermediate	273	29.8 (5.9; 182.3)	23.9 (5.9; 106.9)	58.0 (17.4; 262.4)
High	111	31.0 (6.4; 154.0)	23.0 (5.1; 75.6)	60.4 (17.4; 284.5)
p-value		0.60	0.84	0.72

[a]Low: High school and vocational education or less; Intermediate: High school + 1-3 years; High: High school + 4 years or more.

For the Greenhouse Cohort children, urine samples were obtained at two separate examinations conducted during two different seasons. The first puberty examination was performed between October 17, 2011 and January 18 2012 (BBU2) and the second examination (BBU3) between March 16 and June 17, 2013. At one of the two examinations 128 of the children provided a urine sample after overnight fasting. Table 3.8 shows the pesticide metabolite concentrations after stratifying for season. The concentrations of 3-PBA, 2,4-D, and TCPY were higher in samples obtained in autumn/winter while no difference were apparent for the DAPs.

Table 3.9 shows urinary concentrations of pesticide metabolites according to population characteristics of the Greenhouse Cohort children. The creatinine-adjusted results from non-fasting samples are presented. A weak tendency to higher concentrations in girls than in boys was seen for most of the metabolites although not statistically significant. An age-related decrease was seen for 3-PBA and 2,4-D, and non-significantly for TCPY but not for the DAPs. Socioeconomic status (SES) was grouped into five groups ranked 1(high) to 5 (low) based on parental education and occupation (Hansen 1978) when the women were enrolled in the study. The group of the highest ranked parent living with the child was used. Children from families with high SES had higher 2,4-D concentrations but none of the other pesticide metabolites were markedly related to SES. Children with BMI above the median standard deviation score tended to have lower pesticide metabolite concentrations. Children

whose mothers were still exposed to pesticides by working in greenhouse nurseries tended to have higher 3-PBA concentration while no differences in pesticide concentrations related to residence or home use of pesticides were apparent.

TABLE 3.8. Urinary median concentrations (5; 95 percentiles) of pesticide metabolites in children from the greenhouse cohort according to season of urine sampling

	Fasting samples			**Non-fasting samples**		
	Autumn/Winter (BBU2)	**Spring/summer (BBU3)**	**p-value**	**Autumn/Winter (BBU2)**	**Spring/summer (BBU3)**	**p-value**
N	59	69		96	42	
μg/g creatinine						
3-PBA	0.49 (0.16; 23.3)	0.38 (0.06; 2.65)	0.05	0.59 (0.14; 14.16)	0.28 (0.10; 2.65)	0.0001
2,4-D	0.21 (0.05; 0.83)	0.15 (0.05; 0.82)	0.09	0.28 (0.08; 1.29)	0.18 (0.05; 0.53)	0.006
TCPY	1.55 (0.65; 5.91)	1.25 (0.47; 6.83)	0.06	1.42 (0.20; 9.10)	1.14 (0.30; 2.74)	0.26
nmol/g creatinine						
ΣDMP	NI	NI		41.3 (8.5; 489.8)	49.1 (8.5; 553.7)	0.49
ΣDEP	NI	NI		26.7 (7.2; 184.7)	20.3 (4.6; 101.5)	0.24
ΣDAP	NI	NI		74.2 (19.7; 546.0)	82.0 (15.0; 647.0)	0.87

NI: not investigated

TABLE 3.9 Urinary median concentrations (5; 95 percentiles) of 3-PBA, 2,4-D, TCPY, and DAPs in children (non-fasting) from the greenhouse cohort according to population characteristics

Characteristics	N	3-PBA	2,4-D	TCPY	ΣDMP	ΣDEP	ΣDAP
sex		**µg/g creatinine**			**nmol/g creatinine**		
boys	77	0.41 (0.08; 8.25	0.28 (0.05; 1.00)	1.14 (0.26; 12.41)	39.5 (7.9; 489.6)	20.5 (4.1; 184.6)	66.3 (14.6; 568.6)
girls	63	0.50 (0.12; 8.11)	0.20 (0.07; 1.33)	1.37 (0.26; 7.47)	47.3 (8.4; 469.3)	31.7 (8.0; 143.7)	87.0 (24.1; 504.9)
p-value		0.69	0.34	0.60	0.28	0.10	0.10
Age (years)							
<12	45	0.58 (0.18; 25.69)	0.28 (0.10; 1.68)	1.58 (0.38; 16.04)	39.5 (8.2; 509.4)	28.3 (5.07; 163.7)	68.2 (16.4; 650.0)
12-14	66	0.59 (0.09; 7.30)	0.25 (0.08; 1.29)	1.27 (0.16; 7.24)	43.3 (9.0; 335.6)	20.3 (7.7; 157.4)	73.7 (20.0; 493.3)
>14	29	0.28 (0.08; 5.40)	0.17 (0.04; 0.70)	1.00 (0.28; 6.20)	52.6 (5.5; 795.7)	26.2 (3.7; 188.2)	86.3 (11.0; 858.3)
p-value		0.001	0.05	0.15	0.90	0.52	0.51
SES[a]							
1-3	39	0.32 (0.15; 14.80)	0.32 (0.06; 1.22)	1.14 (0.12; 11.73)	42.4 (8.3; 504.6)	18.3 (4.5; 243.8)	66.3 (14.8; 562.0)
4	68	0.52 (0.18; 4.06)	0.25 (0.08; 1.49)	1.37 (0.22; 5.64)	42.9 (7.8; 253.0)	30.8 (6.78; 168.8)	96.6 (15.2; 347.6)
5	33	0.62 (0.07; 14.39)	0.17 (0.05; 1.02)	1.51 (0.34; 18.81)	45.2 (7.8; 792.9)	24.0 (5.6; 146.4)	68.2 (21.5; 888.2)
p-value		0.23	0.01	0.61	0.97	0.27	0.78
BMI SDS[b]							
≤ median (0.33)	70	0.50 (0.14; 10.34)	0.27 (0.08; 1.04)	1.27 (0.22; 4.29)	52.6 (9.8; 314.6)	22.5 (5.4; 152.3)	86.3 (15.3; 377.5)
> median	68	0.33 (0.09; 7.97)	0.21 (0.05; 1.31)	1.34 (0.25; 10.34)	39.0 (7.7; 494.2)	24.0 (6.8; 177.8)	66.3 (17.1; 610.7)
p-value		0.24	0.37	0.54	0.19	0.89	0.25
Maternal occupational exposure[c,d]							
yes	24	0.62 (0.07; 31.17)	0.24 (0.09; 1.83)	0.95 (0.12; 18.3)	43.2 (7.4; 454.2)	26.6 (4.8; 262.4)	64.1 (15.0; 695.9)
no	95	0.44 (0.11; 6.92)	0.21 (0.05; 1.06)	1.40 (0.32; 6.53)	46.2 (8.2; 502.0)	22.2 (6.1; 137.2)	83.6 (16.0; 555.9)
p-value		0.19	0.48	0.11	0.52	0.53	0.73
Residence[d]							
Urban/suburban	42	0.41 (0.08; 4.57)	0.21 (0.05; 1.67)	1.37 (0.29; 6.43)	43.1 (7.1; 502.1)	24.2 (6.5; 187.1)	99.1 (14.5; 654.7)
Rural	78	0.50 (0.15; 14.08)	0.23 (0.08; 1.14)	1.18 (0.28; 6.86)	45.0 (7.9; 501.7)	21.8 (4.5; 114.3)	71.2 (15.6; 521.7)

p-value		0.66	0.80	0.71	0.59	0.65	0.47
Residential pesticide use (in house) [d]							
yes	49	0.36 (0.08; 7.45)	0.20 (0.07; 0.78)	1.14 (0.38; 6.28)	49.7 (9.4; 499.7)	21.8 (6.1; 177.8)	83.7 (20.1; 585.6)
no	66	0.55 (0.13; 11.8)	0.27 (0.06; 1.58)	1.34 (0.28; 7.94)	40.9 (7.7; 502.9)	22.5 (6.7; 135.6)	70.2 (14.8; 618.1)
p-value		0.40	0.27	0.88	0.26	0.93	0.30

[a] SES: Socioeconomic status (social class 1-3/4/5); [b] BMI SDS: age and sex specific BMI Standard Deviation Score; [c] mother current working in greenhouse nursery with pesticide use; [d] questionnaire data from the second puberty examination (BBU3) including 133 children.

3.3 Comparison of urinary pesticide concentrations to other studies

Previous studies measuring urinary concentrations of 2,4-D and organophosphate and pyrethroid metabolites were identified using the PubMed database. We limited the search to studies published after 2000 and based on non-occupationally exposed populations. The primary focus of interest was pregnant women and children but studies reporting on levels in the general population were included as well. We decided to report and compare the unadjusted concentrations, as these were available in most studies.

Table 3.10 present studies reporting urinary 3-PBA concentrations as marker of pyrethroid exposures. The concentration of 3-PBA in urine from the women from the OCC was higher than in the PELAGIE cohort from France and the CHAMACOS cohort from the USA but similar to or lower than levels found in adults in other cohorts. However, the concentrations measured in the OCC might be underestimated because the women were fasting at sampling. In contrast, the 3-PBA concentration found in the Greenhouse cohort children were higher than in most other studies on children except the MICASA study among farm worker families in the US. The concentration measured at this examination of the children was similar to the concentration measured when the children were between 6 and 11 years old.

The TCPY concentration measured in the OCC women was higher than concentrations measured in Norway and the Netherlands and also higher than in the general adult population in the US as found in The National Health and Nutrition Examination Survey (NHANES) (Table 3.11). The concentration was lower in the OCC than in the CAMACHOS study among farm worker families in California and the Mt. Sinai study where chlorpyrifos had been used for residential insect control. Also the TCPY concentration in children was high compared to most other studies on children except for two studies from Spain and Australia, respectively, reporting markedly higher concentrations.

Regarding the total organophosphate insecticide exposure, as estimated by the urinary ΣDAP concentration, the women from the OCC had lower level than seen in most of the other studies including the Danish part of DEMOCHOPHES in which DAPs were measured in urine samples collected within the same period as the samples in OCC (Table 3.12). The women in DEMOCHOPHES delivered first-morning-voids, known to be more concentrated than spot urine samples and this, combined with the fasting state of the women in OCC, might explain some of the concentration difference. Comparing the creatinine adjusted results did, however, only slightly diminish the difference (medians in nmol/g creatinine: OCC 58.4 and DEMOCHOPHES: 88.1). The women in the French PELAGIE cohort had lower ΣDAP concentration than the Danish women. For the Greenhouse children, the ΣDAP concentration was lower at this examination than at the examination in 2007-08, and the concentration was also slightly lower than for the DEMOCHOPHES children although the difference was less than for the women. The concentration of ΣDAPs in the Greenhouse Cohort children seem to be higher than levels reported from children included in NHANES and rather similar to levels found in children from the CHAMACOS study in the US and in the Canadian CHMS study.

Only few studies have investigated urinary concentrations of 2,4-D in non-occupational exposed populations. The concentrations measured in women and children in our study were lower than the levels reported in both adults and children from NHANES and in a study from Ohio, but similar to levels found in the CHAMACOS cohort and in a study from Puerto Rica although a higher LOD in these studies hampered the comparison (Table 3.13).

TABLE 3.10: Comparison of urinary concentrations of the generic pyrethroid metabolite, 3-PBA, with concentrations found in other studies. The values represent volume based concentrations (μg/L) in spot urine samples unless otherwise stated.

Study	Population	Sampling year	N	LOD	%>LOD	GM	50th pct (median)	95th pct	Remark	Ref
Europe										
OCC, Denmark	Pregnant Women	10-12	858	0.03	94.3	0.22	0.20	2.18	Fasting, GW 28	This study
Greenhouse Cohort Children,	Children 10-16 yrs	11-13	143	0.03	100	0.66	0.56	8.90	Non-fasting	This study
	Children 10-16 yrs		128		100	0.51	0.49	8.98	Fasting	
Greenhouse Cohort Children,	6-11 yrs	07-08	173	0.8	41.0	0.66	<LOD	4.11	first-morning-voids	Andersen et al. (2012b)
PELAGIE, France	Pregnant women	02-06	205	0.008	30.2	-	<LOD	-	first trim, first-morning-voids	Viel et al. (2015)
	Children, 6 yrs		284		63.7	-	0.018	-		
GerES, Germany,	Children 3-14 yrs	03-06	598	0.1	98	-	0.43	3.80		Schulz et al. (2009)
Poland	All	12	374	0.1	82.4	0.26	0.25	1.24		Wielgomas and Piskunowicz (2013)
	<18 yrs		184		-	0.29	-	-		
	>18 yrs		190		-	0.23	-	-		
Spain	Children 6-11 yrs	10		0.8	23	-	<LOD	12.33*	*μg/g creatinine	Roca et al. (2014)
America										
NHANES, USA	Children 6-11 yrs	09-10	383	0.1		0.55	0.48	8.51		CDC (2015)
	20-59 yrs		1296			0.42	0.39	6.95		
NYC HANES, USA	>20 yrs	04	1452	0.64	58.5	-	0.76	5.23		McKelvey et al. (2013)
SUPERB, USA	Children 2-8 yrs	07-09	83	0.75	60		1.56	4.69	Residential use	Trunnelle et al. (2014b)

	18-57 yrs		64		90		1.58	9.44		
MICASA, USA	Children 2-8 yrs Mothers 23-52 yrs	09	103 105	0.1	78 82	1.11 1.17	1.93 1.63	7.36 13.29	Farm worker families	Trunnelle et al. (2014a)
CHAMOCOS, U.S.	Pregnant Women	99-01	481	0.1	27	-	<LOD	1.1	Agricultural area, Second trim, GW 26	Castorina et al. (2010)
Mt. Sinai, New York, USA	Pregnant women	98-01	307			-	18.3	126.9*	Third trim, *90th pct Residential use	Berkowitz et al. (2003)
CHMS, Canada	Children 6-11 yrs All, 6-79 yrs	07-09	1032 5604	0.01	99.3 99.4	 0.25	0.20 0.23			Oulhote and Bouchard (2013); Ye et al. (2015)
ELEMENT, Mexico	Pregnant women	97-01	187	0.25	56	0.26	<LOD	0.85	third trimester	Watkins et al. (2016)
Caribbean	Pregnant women	08-11	297	0.01	100	0.54	-	3.51	third trimester	Dewailly et al. (2014)
PROTECT, Puerto Rico	Pregnant Women	10-12	54	0.1		0.2	<LOD	2.3	second trimester	Lewis et al. (2014)
Asia										
Japan	Pregnant Women	09-11	231	0.02	97.8	0.33	0.35	-	GW 10-12	Zhang et al. (2013)
China	Pregnant Women	10-12	322	0.1	82	0.37	0.50	2.6		Ding et al. (2015)

GM: geometric mean; GW: gestational week

TABLE 3.11 Comparison of urinary concentrations of the specific metabolite of chlorpyrifos/chlorpyrifos-methyl,TCPY, with concentrations found in other studies. The values represent volume based concentrations (μg/L) in spot urine samples unless otherwise stated.

Study	population	Sampling year	LOD	%>LOD	N	GM	50th pct (median)	95th pct	Remark	Ref
Europe										
OCC, Denmark	Pregnant women	10-12	0.3	93.2	858	1.67	1.74	8.15	Fasting, GW 28	This study
Greenhouse Cohort Children	Children10-16 yrs	11-13	0.3	95.8	143	1.42	1.43	6.05	Non-fasting	This study
				94.5	128	1.52	1.55	7.31	Fasting	
MoBa, Norway	Pregnant women	99-04			10*	0.99			second trim, *pooled samples	Ye et al. (2009)
Generation R, The Netherlands	Pregnant women	04-06	0.15	100	100	1.2	1.2	6.4	> GW 20	Ye et al. (2008)
Spain	Children 6-11 yrs	10	0.80	86	125	3.36*	3.40*	12.97*	*μg/g creatinine	Roca et al. (2014)
America										
NHANES, USA	Children 6-11 yrs	09-10	0.1		386	1.12	1.46	5.81		CDC (2015)
	Adults 20-59 yrs				1309	0.71	0.97	4.18		
CHAMOCOS, USA	pregnant women	99-01	0.3	81.9	481	-	3.2	17.9	Agricultural area, Second trim, GW 26	Castorina et al. (2010)
Mt. Sinai, New York, USA	pregnant women	98-01			365	-	7.5	61.2*	Third trim, *90th perc Residential use	Berkowitz et al. (2003)
ELEMENT, Mexico City	pregnant women	97-05	0.1	>90	187	1.76	1.78	11.6	Third trim	Fortenberry et al. (2014)
Puerto Rico	Pregnant women	10-12	0.1	86.2	54	0.4	0.5	2.0	4 samples per women	Lewis et al. (2015)
Australia	Children 2.5-6 yrs	03-06		92.2	115	-	12.5*	71.1*	*μg/g creatinine	Babina et al. (2012)
Asia										
China	Children 3-6 yrs	2014		44.1	406	0.92*	0.63*	22.9*	*μg/g creatinine	Wang et al. (2016)

GM: geometric mean; GW: gestational week

TABLE 3.12 Comparison of urinary concentrations of total dialkyl phosphate metabolites (ΣDAP) in samples from this study with concentrations found in other studies. The values represent volume based concentrations (nmol/L) in spot urine samples unless otherwise stated.

Study	Population	Sampling Year	N	GM	50th pct (median)	95th pct	Remark	Ref.
Europe								
OCC, Denmark	Pregnant women	10-12	564	58.7	56.5	253	Fasting, GW 28	This study
Greenhouse Cohort Children,	Children10-16 yrs	11-13	141	89.7	85.6	506		This study
Greenhouse Cohort Children,	Children 6-11 yrs	07-08	172	160.4	153.7	1252	First-morning-voids	Andersen et al. (2012b)
DEMOCHOS, Denmark	Pregnant women	11	145	84.8	92.3		First-morning-voids	Mørck et al. (2016)
	Children 6-11 yrs		144	111	106			
PELAGIE	Pregnant women	02-06	254		38.8		First-morning-voids	Debost-Legrand et al. (2016)
Generation R, Holland	Pregnant women	04-06	100	183	200	659	GW 20	Ye et al. (2008)
MoBa. Norway	Pregnant women	99-04	10	87*			10 pools, each consisting of pooled urine from 11 women * Calculated from μg/L	Ye et al. (2009)
Greece, Crete	Adults	08-09	86	-	15	-	Agricultural area	Kokkinaki et al. (2014)
America								
NHANES, USA	Children 8-15 yrs	00-04	1139	68.3				Bouchard et al. (2010)
HOME, Ohio, USA	Pregnant women	03-06	327	73.7*	96.7*		*nmol/g creatinine, two spot urine samples during preg,	Donauer et al. (2016)
NYC HANES, USA	Adults >20 yrs	04	876	-	114.9	1321.8		McKelvey et al. (2013)
Mount Sinai, USA	Pregnant women	98-02	342	75.5*	77.9*	894.7*	*nmol/g creatinine , residential use	Harley et al. (2016)
CHAMACOS, U.S.	Pregnant women	99-00	348	109	-	-		Marks et al. (2010)
	Children 5 yrs	04-05	320	92.6				
MIREC, Canada	Pregnant women	08-11	1884	78	78	538	First trim	Sokoloff et al. (2016)

CHMS, Canada	Children 6-11 yrs	07-09	1035	-	99.2	-	Oulhote and Bouchard (2013); Ye et al. (2015)
	All, 6-79 yrs		5604	76.7	71.4		

GM: geometric mean; GW: gestational week

TABLE 3.13 Comparison of urinary concentrations of the chlorophenoxy herbicide 2,4-D with concentrations found in other studies. The values represent volume based concentrations (ug/L) in spot urine samples unless otherwise stated.

Study	Population	Sampling year	N	LOD	%>LOD	GM	50th pct (median)	95th pct	Remark	Ref
Europe										
OCC, Denmark	pregnant women	10-12	858	0.03	97.6	0.16	0.16	0.70	Fasting, GW 28	This study
Greenhouse Cohort Children,	10-16 yrs	11-13	141 128	0.03	100 99.2	0.26 0.18	0.25 0.19	1.04 0.77	Non-fasting Fasting	This study
Spain	6-11 yrs	10	125	0.40	9	-	<LOD	0.43*	*μg/g creatinine	Roca et al. (2014)
America										
NHANES, USA	6-11 yrs 20-59 yrs	09-10	386 1309	0.15		0.385 0.288	0.350 0.270	1.59 1.33		CDC (2015)
CHAMACOS, USA	pregnant women	99-01	481	0.2	21.2	-	<LOD	0.8	Agricultural area, Second trim, GW 26	Castorina et al. (2010)
Ohio, USA	20-49 yrs Preschool children	01 00-01	121 66	0.2	89 97		0.7 1.2	3.1 4.3	4-6 samples in 48 h	Morgan et al. (2008); Morgan (2015)
PROTECT, Puerto Rico	pregnant women	10-12	54	0.4			<LOD	0.6	second trim.	Lewis et al. (2014)

GM: geometric mean; GW: gestational week

3.4 Prenatal and current pesticide exposure and motor speed function and attention in children

At the first follow-up examination of the Greenhouse Cohort children in 2007-08, when they were between 6 and 11 years old, a battery of neurodevelopmental tests were included to examine a broad spectrum of functions. We found impaired neuropsychological function in girls, but not boys, whose mothers were occupationally exposed to pesticides in early pregnancy (Andersen et al. 2015). Especially language- and motor speed functions in girls were significantly inversely associated with prenatal pesticide exposure. We did not have the possibility to repeat the whole testing battery in this study, but two of the tests investigating motor speed function (Finger tapping Test) and attention (Conners' Continuous Performance Test II) were included in the last examination performed in 2013. We wanted to investigate if associations with prenatal pesticide exposure were still apparent and if the children's own current exposure levels were related to these outcomes.

Table 3.14 shows the results for the motor speed function for all children and for boys and girls separately. We did not find any associations between the children's current urinary pesticide metabolite concentrations and motor speed function. However, girls whose mothers were occupationally exposed to pesticides in pregnancy tended to have decreased motor speed function although not statistically significant at the 0.05-level. This finding confirms the results from the first follow-up examination when the children were between 6 and 11 years and may indicate a permanent impairment of this function in the girls after prenatal exposure. We do not have biomonitoring data from the mothers in pregnancy and therefor, specific pesticides related to this finding cannot be identified.

In Table 3.15 results from the computer test of attention are shown for the children. More omission errors (missed responses) were seen for prenatally exposed children. None other associations were apparent in the whole group of children neither in relation to prenatal or the children's current pesticide exposure. However, in sex-stratified analysis the current exposure to organophosphates (urinary concentrations of TCPY and DAPs) were associated with a higher number of omission errors in girls. The associations were significant for TCPY, ΣDMP, and ΣDAP (Figure 3.1). A tendency to more commission errors was also seen among the girls in relation to current organophosphate exposure. No sexually dimorphic associations were seen for average reaction time.

TABLE 3.14 Test of manual motor speed function. The values shown are adjusted[a] mean score difference (β) with 95% confidence intervals (95% CI), for the Finger tapping test in relation to prenatal and current pesticide exposure in children from the Greenhouse Cohort. The results present the average for both hands (mean number of taps in 10 seconds for two trials with dominant and two trials with non-dominant hand).

	All children			**Boys**			**Girls**		
	N	β (95% CI)	p-value	N	β (95% CI)	p-value	N	β (95% CI)	p-value
Prenatal exposure[b]	130	-1.19 (-3.51; 1.13)	0.31	72	0.57 (-2.46; 3.60)	0.71	58	-3.35 (-7.09; 0.40)	0.08
Current exposure[c]									
3-PBA	119	0.15 (-1.85; 2.16)	0.88	66	0.90 (-1.92; 3.73)	0.53	53	-0.69 (-3.76; 2.38)	0.65
2,4-D	119	-0.53 (-3.51; 2.46)	0.73	66	1.15 (-2.88; 5.19)	0.57	53	-2.76 (-7.78; 2.26)	0.27
TCPY	121	-0.74 (-3.40; 1.93)	0.59	67	-0.49 (-3.80; 2.62)	0.71	54	-0.10 -6.04; 5.83)	0.97
ΣDMP	120	0.40 (-1.74; 2.54)	0.71	67	0.39 (-2.36; 3.13)	0.78	53	0.32 (-3.33; 3.97)	0.86
ΣDEP	120	0.55 (-2.18; 3.28)	0.69	67	1.46 (-2.13; 5.06)	0.42	53	-0.47 (-4.94; 4.00)	0.83
DΣAP	120	0.51 (-2.04; 3.07)	0.69	67	0.63 (-2.58; 3.83)	0.70	53	0.35 (-4.11; 4.82)	0.87

[a] Adjusted for age (in years) at examination, SES, and for all children also child sex. [b]score difference between prenatally exposed and unexposed (yes/no);
[c] score difference for a 10-fold increase in the urinary (creatinine adjusted) concentration of the pesticide metabolite

TABLE 3.15 Corners Performance test of attention. The values shown are adjusted[a] mean test score difference (β) with 95% confidence intervals (95% CI) in relation to prenatal and current pesticide exposure in children from the Greenhouse Cohort.

			Reaction time (ms), average of 15 min		**Number of omission errors**		**Number of commission errors**	
		N	β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value
Prenatal exposure[b]	All	130	7.0 (-7.5; 21.6)	0.34	2.60 (0.40; 4.81)	0.02	1.02 (-1.70; 3.73)	0.46
Current exposure[c]								
3-PBA	All	119	-6.1 (-19.4; 7.2)	0.36	1.45 (-0.59; 3.50)	0.16	0.44 (-2.00; 2.87)	0.72
2,4-D	All	119	-4.7 (-24.5; 15.1)	0.64	0.55 (-2.52; 3.62)	0.72	1.27 (-2.35; 4.89)	0.49
TCPY	All	121	-12.7 (-29.8; 4.4)	0.15	1.45 (-1.19; 4.19)	0.28	1.06 (-2.10; 4.21)	0.51
ΣDMP	All	120	5.28 (-8.44; 19.00)	0.45	1.28 (-0.84; 3.40)	0.24	0.73 (-1.81; 3.26)	0.57
ΣDEP	All	120	-0.31 (-17.85; 17.23)	0.97	0.79 (-1.93; 3.50)	0.57	0.61 (-2.62; 3.84)	0.71
ΣDAP	All	120	3.5 (-12.86; 19.91)	0.67	1.48 (-1.05; 4.01)	0.25	1.09 (-1.92; 4.10)	0.48

[a] Adjusted for age (in years) at examination, SES, and child sex. [b]score difference between prenatally exposed and unexposed (yes/no); [c] score difference for a 10-fold increase in child urinary concentration (µg/g creatinine) of the pesticide metabolite.

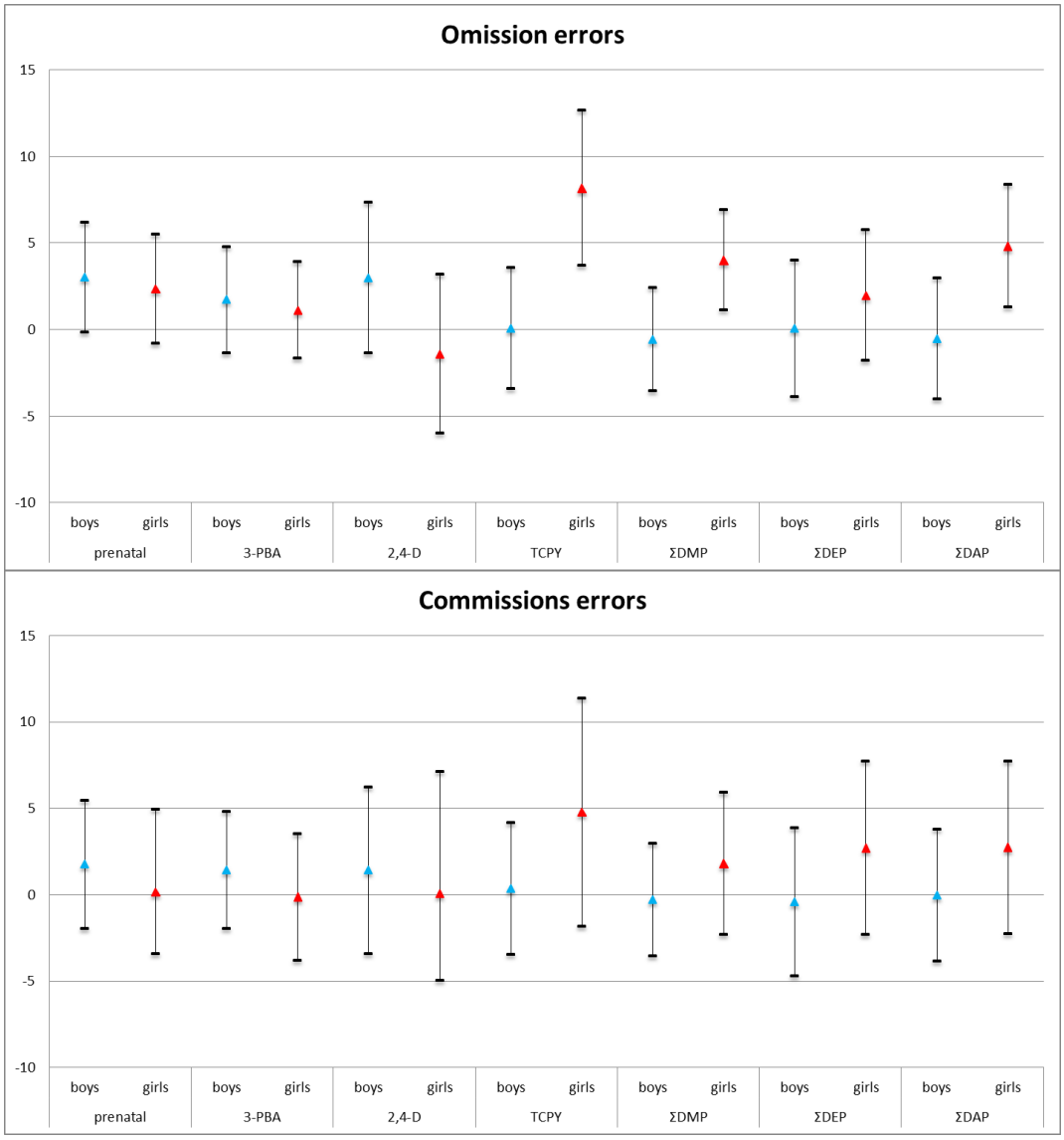

FIGURE 3.1 Mean differences in number of omission (missed responses) and commission (false positives) errors for prenatally exposed children compared to unexposed children and for a 10-fold increase in current pesticide exposure (urinary metabolite concentrations (µg/g creatinine).

3.5 Maternal urinary pesticide metabolite concentrations and birth outcomes and ano-genital distance (AGD) in the offspring at three months of age

The results from this sub-study are presented in paper 2. The main findings are presented below.

The aim of this study was to investigate whether maternal pregnancy urinary concentrations of 2,4-D and organophosphate and pyrethroid insecticide metabolites were associated with birth outcomes (gestational length, birth weight, head and abdominal circumference) and AGD measured three months after expected date of birth.

We did not find any consistent dose-related associations between maternal pesticide metabolite concentrations and gestational length, birth weight, head or abdominal circumference, although some tendencies appeared. Hence, gestational length tended to be longer for boys in the third tertile of ΣDAP compared to the boys in the first (3.2 (95% CI: 0.1; 6.4) days, p-trend: 0.05). Further, a tendency towards a smaller abdominal circumference with increasing 3-PBA concentrations was seen in girls (β: -0.3 (95%CI: -0.5; -0.003) cm, p trend: 0.05).

We did not find any clear dose-response relationship between the maternal urinary concentrations of pesticide metabolites and AGD in the offspring (Figure 3.2 and Figure 3.3). However, a tendency towards a longer AGD with higher maternal concentrations of 3-PBA (AGDac, p-trend: 0.14) and ΣDE (AGDac, p-trend: 0.08) was seen in girls. Further, a statistically significant shorter AGD in boys with maternal concentrations of 2,4-D in the second compared to the first tertile (AGDas: -1.55 (95% CI: -2.81, -0.28) mm and AGDap: -1.62 (95% CI: -3.00, -0.24) mm) was found. Thus, the results might suggest a weak sexually dimorphic effect.

The tendency to a longer AGD in girls related to increased ΣDE concentration was not reflected in a similar tendency for TCPY despite chlorpyrifos is metabolized to TCPY and DEP. This may indicate that the combined exposure to ethylated organophosphates was necessary to induce a weak effect. To investigate potential interaction between the different groups of pesticides on AGD, we performed pair-wise interaction analyses of those pesticides measured in all the 858 mother-child pair (i.e., 2,4-D, TCPY, and 3-PBA). We used categorical variables of the pesticides (above or below the medians). The results are shown in Table 3.16 and Table 3.17. We did not see any indication of additive effects in these rather crude analyses.

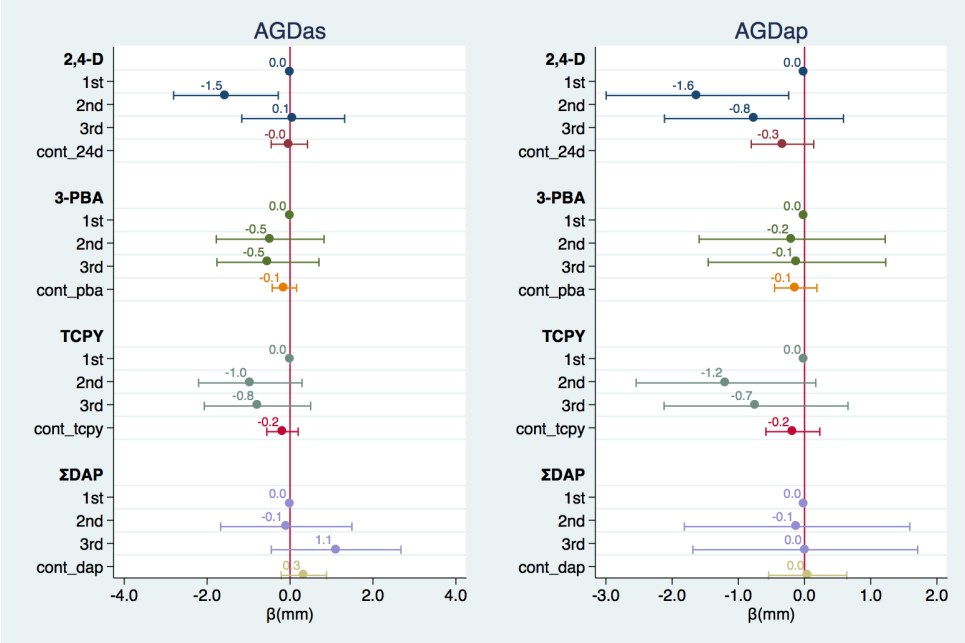

FIGURE 3.2 Results of multiple linear regression analyses (β) of AGD in boys and maternal urinary pesticide metabolite concentrations (µg/g creatinine) divided into tertiles (β = change in AGD in mm compared to 1st tertile) and as continuous variables (β = change in AGD in mm when doubling exposure)

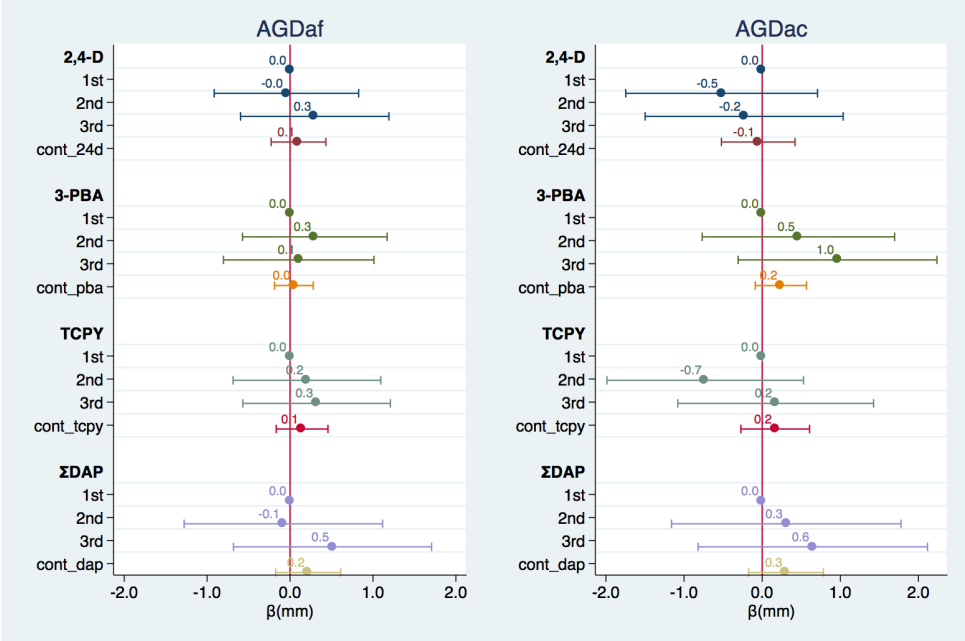

FIGURE 3.3. Results of multiple linear regression analyses of AGD in girls and maternal urinary concentrations (µg/g creatinine) of pesticide metabolites divided into tertiles (β = change in AGD in mm compared to 1st tertile) and as continuous variables (β = change in AGD in mm when doubling exposure)

TABLE 3.16. Estimated change (mm) in AGDas and AGDap (with 95% CI) in boys after combined prenatal exposure to 2,4-D and 3-PBA, 2,4-D and TCPY or 3-PBA and TCPY estimated by maternal urinary metabolite concentrations (µg/g creatinine).

	AGDas				AGDap			
	3-PBA		TCPY		3-PBA		TCPY	
	<median	≥ median	< median	≥ median	< median	≥ median	< median	≥ median
2,4-D								
< median	ref	-1.06 (-2.55; 0.44)	ref	0.13 (-1.38; 1.63)	ref	-1.31 (-2.94; 0.32)	ref	0.35 (-1.29; 2.00)
≥ median	-0.92 (-2.39; 0.54)	-0.63 (-2.07; 0.80)	0.58 (-0.86; 2.03)	-0.82 (-2.18; 0.55)	-1.92** (-3.50; -0.35)	-1.09 (-2.64; 0.46)	-0.12 (1.69; 1.46)	-1.17 (-2.65; 0.31)
3-PBA								
< median			ref	-1.43* (-2.89; 0.03)			ref	-0.96 (-2.54; 0.62)
≥ median			-1.06 (-2.50; 0.38)	-1.03 (-2.46; 0.41)			-0.63 (-2.21; 0.93)	-0.67 (-2.23; 0.89)

*p<0.1; **p<0.05

TABLE 3.17 Estimated change (mm) in AGDaf and AGDac (with 95% CI) in girls after combined prenatal exposure to 2,4-D and 3-PBA, 2,4-D and TCPY or 3-PBA and TCPY estimated by maternal urinary metabolite concentrations (µg/g creatinine).

	AGDaf				AGDac			
	3-PBA		TCPY		3-PBA		TCPY	
	< median	≥ median	< median	≥ median	< median	≥median	<median	≥median
2,4-D								
< median	ref	0.13 (-0.86; 1.12)	ref	0.46 (-0.53; 1.46)		1.45** (0.06; 2.84)		0.09 (-1.32; 1.50)
≥ median	0.63 (-0.39; 1.66)	0.16 (-0.89; 1.22)	1.01 (-0.08; 2.10)	0.26 (-0.67; 1.19)	0.63 (-0.82; 2.08)	1.06 (-0.43; 2.54)	0.60 (-0.95; 2.16)	-0.19 (-1.50; 1.13)
3-PBA								
<p50			ref	-0.23 (-1.26; 0.81)				-0.60 (-2.05; 0.86)
≥p50			-0.43 (-1.48; 0.63)	-0.15 (-1.11; 0.81)			0.88 (-0.61; 2.36)	0.60 (-0.74; 1.95)

*p<0.1; **p<0.05

4. Discussion

4.1 Prenatal pesticide exposure and epigenetic

In the Greenhouse Cohort children, we found that maternal occupational pesticide exposure in early pregnancy was associated with a differential methylation profile in DNA isolated from blood samples from exposed children carrying the *PON1* 192R-allele compared to children with the *PON1* 192QQ genotype and unexposed children. Some of the differentially methylated genes are known to be involved in neuroendocrine pathways that regulate appetite and energy homeostasis. Since the methylation profile was measured in blood samples obtained at the same time as the health outcomes at age 6 - 11 years, we cannot prove that the link is causal. Hence, the differentially methylated positions might be a consequence of alterations of food habits and physical activity among the exposed children with the PON1 192R-allele and not an underlying mechanism. However, the mediation analysis performed suggested that at least some of the differentially methylated marks are on the mechanistic pathway between prenatal pesticide exposure and higher body fat content and leptin levels. Furthermore, the association was strongest between pesticide exposure and delta BMI Z-score that integrates fat accumulation from birth and onwards to school age. Thus, our findings do suggest a link between epigenetics and genetic susceptibility towards pesticide exposure in foetal life (see paper 1 for a more comprehensive discussion of the results).

4.2 Urinary concentrations of pesticide metabolites

The results from this study demonstrate a widespread exposure to organophosphate and pyrethroid insecticides and to the chlorophenoxy herbicide 2,4-D in the Danish population, as metabolites were detectable in urine samples from more than 90% of the women and children in this study. The exposure levels for both groups of insecticides, pyrethroids in particular, were similar or higher among the children in this study than in most other published studies on populations without particularly residential exposure. This might be of concern since some recent studies reported associations between urinary concentrations of 3-PBA in children and impaired cognitive functions (Viel et al. 2015) and increased risk of behavioural problems (Oulhote and Bouchard 2013; Viel et al. 2017) including attention-deficit hyperactive disorder (ADHD) (Wagner-Schuman et al. 2015) at exposure levels below those found among the children in this study. The cross-sectional design of these studies does not allow conclusions about causality but emphasise the need for longitudinal follow-up of studies of children with childhood exposure data. Besides, associations between prenatal exposure to some insecticides, including pyrethroids, and impaired attention and other neurobehavioral outcomes have also been reported from longitudinal birth cohort studies (Abreu-Villaca and Levin 2017; De Felice et al. 2014; Fluegge et al. 2016; Furlong et al. 2017a; Furlong et al. 2017b; Viel et al. 2017).

The exposure levels among the pregnant women from the OCC tended to be lower than in most other studies although for chlorpyrifos, the metabolite (TCPY) concentration was only higher for pregnant women with agricultural or residential exposures. Furthermore, the exposure levels for the OCC women might be underestimated because the spot urine samples were obtained after over-night fasting while the other studies used non-fasting spot urine samples. Comparisons of exposure levels between studies rely on pesticide metabolite concentrations measured at different laboratories and minor deviations in the exact values cannot be ruled out. However, we consider the levels to be comparable because the laboratories in general use established and validated methods, participate in ring test (when available), and use external as well as internal quality control samples and high quality standards.

In our study, the pesticide metabolite concentrations were determined in one single spot urine sample and this procedure has some limitations. The pesticides are assumed to be rapidly metabolized and to be excreted from the body within hours to days and substantial within-subject variability has been demonstrated for organophosphate metabolites (Spaan et al. 2015) and to a lesser degree for pyrethroid metabolites (Watkins et al. 2016; Wielgomas 2013). While within-subject variation is unlikely to affect comparison of exposure levels between different studies, it might have serious impact for investigating associations between exposure level and health outcomes. In the OCC study of AGD and birth outcomes (paper 2), we tried to reduce potential exposure misclassification due to this day-to-day variation by dividing the continuous exposure variables into categorical variables (tertiles) assuming that individuals with a general high intake of non-organic fruit and vegetables are more likely to be in the upper tertile compared to those with lower intake. This assumption is supported by a previous study, showing that a single urine measure of TCPY was valid to predict tertiles of exposure (Meeker et al. 2005). Compared to DAPs and TCPY, the individual levels of 3-PBA were reported to be more stable (Wielgomas 2013). Most pyrethroids are highly lipophilic and finding of relatively high concentrations of these substances in human breast milk have raised concern that they are not always so rapidly excreted as earlier anticipated (Corcellas et al. 2012). Further, the use of urinary pesticide metabolite concentrations as markers of exposure is often criticised with the argument that it overestimate the exposure to the parent pesticide because preformed metabolites in the diet will also be included in the measurement. Thus, comparison of urinary metabolite concentrations between studies with different exposure pathways might, to some extent, be misleading. However, some pyrethroid metabolites, e.g, 3-PBA, have been demonstrated to exhibit anti-androgenic activity *in vitro* with similar or even greater potencies that the parent compounds (Saillenfait et al. 2016a; Tyler et al. 2000). Thus, preformed metabolites in the diet should probably not always be considered as nontoxic or less toxic than the parent compound. For that reason, the toxicity and exposure of a metabolite is also considered when establishing the residue definition. Moreover, we cannot exclude that some other chemicals may contribute to the measured urinary metabolites, especially the unspecific DAPs and 3-PBA. However, urinary metabolite concentrations have been shown to be markedly reduced after one week of limiting consumption to organic food (Bradman et al. 2015; Oates et al. 2014) and to be related to frequent consumption of organic produce reported by questionnaire in population studies (Curl et al. 2015). Thus, pesticide residues in food items, especially fruit and vegetables are consistently found to be the main predictor for the concentrations of these metabolites in urine samples from the general population (Spaan et al. 2015; Ye et al. 2015), although residential use and proximity to agricultural use might contribute to a minor degree (Lind et al. 2017). In our study, the urinary concentrations of pesticide metabolites among the greenhouse cohort children were unrelated to residence and home-use of pesticides. However, a tendency towards higher 3-PBA concentrations in children whose mothers were still working in greenhouse floricultures may indicate some take-home exposure to pyrethroids.

The high detection frequency of 2,4-D among both the Greenhouse Cohort children and women from the OCC was unexpected. It is most likely related to an increasing use of 2,4-D as growth regulator in citrus fruit production (Chen et al. 2015). Accordingly, urine samples collected from the children during autumn/winter had a higher 2,4-D concentration than samples collected during spring/summer. This might reflect a higher intake of citrus fruit in the later part of the year. Also for 3-PBA, the median urine concentration was higher in samples collected during autumn/winter but potential food items responsible for this difference is not obvious and the finding could also be due to other sources than diet. Since no organophosphate insecticides are allowed for use in food production in Denmark, the relatively high urine concentrations of TCPY and DAPs found in this study must reflect exposure from imported fruit and vegetables. According to the 2016 EFSA report on pesticide residues in food samples collected in 2014 in the EU (European Food Safety Authority 2016) pesticide residues were detected in 45.3 % of the conventional food products (3.0 % above the maximum residue level (MRL). Residues of more than one pesticide were found in 28.3% of the samples. Similar findings

were seen in the Danish Pesticide Residue Monitoring Programme (Fødevareinstituttet 2016). Chlorpyrifos was among the most frequently detected pesticides and the one that most often exceeded the acute reference dose (ARfD) in the EU-survey reported in 2015 ((European Food Safety Authority 2015). Thus, despite chlorpyrifos has never been allowed in food production in Denmark, the population seem to be widely exposed from imported fruit and vegetables. At the same time a high intake of fruit and vegetables is related to a healthy dietary lifestyle (Groth et al. 2001) and this might explain the higher urinary concentration of organophosphate metabolites and 2,4-D seen among the most well-educated and slim women from the OCC in this study. In contrast, the urinary concentration of 3-PBA did not follow the same distribution pattern in relation to population characteristic as the other pesticides. The higher concentration in nulliparous women and women with a pre-pregnancy BMI above 25 kg/m^2 might indicate that, at least some pyrethroids are metabolized and excreted more slowly than previously assumed and/or that the dietary sources are different than for organophosphates and 2,4-D. Pyrethroids are lipophilic substances, and they have been detected in human breast milk samples, sometimes in relatively high concentrations (Corcellas et al. 2012). Furthermore, the concentration in human milk samples was reported to be inversely associated with number of pregnancies and thus in accordance with or finding of lower urine concentration of 3-PBA in multiparous women.

4.3 Pesticide exposure and health outcomes in the Greenhouse Cohort children

Although the children in this study had relatively high urine concentrations of 3-PBA compared to other studies on children, we did not observe any associations between the children's current urinary 3-PBA concentration and motor speed function or attention. Other recent studies reported associations between impaired neurodevelopment or behavioural difficulties and the children's current urine concentrations of pyrethroid metabolites (Oulhote and Bouchard 2013; Viel et al. 2015; Viel et al. 2017; Wagner-Schuman et al. 2015) even at lower 3-PBA concentrations than found in our study. The few neurodevelopmental outcomes included in our study do only investigate specific functions, i.e., manual motor speed function and attention. These tests might not be sensitive enough to detect an effect in a relatively small group of children and other brain functions not covered by these tests might be affected. Thus, a more comprehensive testing battery combined with a longitudinal study design are needed to draw conclusion on potential neurodevelopmental effects of pyrethroids at the current exposure level in children.

Our results showed a positive association between concurrent organophosphate exposure and number of hit errors in the attention test for girls but not for boys. This is in line with our previous findings from this cohort in relation to prenatal pesticide exposure where neurodevelopment seemed more affected in girls than in boys (Andersen et al. 2015). In an experimental study in mice, gestational exposure to chlorpyrifos (6 mg/kg/day per os on GD 14-17) was demonstrated to cause sex-dimorphic neurodevelopmental effects with females being most sensitive (De Felice et al. 2014). Some studies in humans found boys to be more affected than girls after prenatal or postnatal organophosphate exposure (Horton et al. 2012; Marks et al. 2010) although in most studies the data analyses were adjusted for sex but potential sex-differences in neurodevelopmental outcomes were not investigated. A recent study, based on the French PELAGIE Cohort, found a negative association between the children's urine concentrations of diethyl phosphate metabolites, ΣDEP, and their working memory score at 6 years of age (Cartier et al. 2016) at concentrations considerably below those measured in the Greenhouse Cohort children in this study. The median of ΣDEP among the children in the PELAGIE cohort was 0.8 nmol/L versus 24.1 nmol/L in the Greenhouse Cohort children. As for the pyrethroids, longitudinal studies are warranted to further investigate potential effects on the nervous system of childhood exposure to organophosphate insecticides at the current exposure levels.

4.4 Prenatal pesticide exposure, birth outcomes, and anogenital distance at 3 months in the OCC

In accordance with other birth cohort studies among women without occupational or high residential pesticide exposure (Harley et al. 2016), we did not find indications on adverse effects on birth outcomes. A slight reduction in abdominal circumference in female new-borns associated with maternal urinary concentration of 3-PBA and a higher gestational age at birth in boys associated with ΣDAP concentrations was seen but the findings were not consistent and might be random findings.

We did not observe any significant dose-related associations between maternal urinary concentrations of the pesticide metabolites and AGD in the infants. However, weak sexually dimorphic associations were indicated, as 3-PBA and ∑DE tended to be dose-related to a longer AGD in girls and as boys in the intermediate tertile of 2,4-D compared to the first tertile had shorter AGDs. Since no change was seen from the first to the third tertile of 2,4-D, this finding is likely due to chance, e.g. caused by multiple comparisons or it might be influenced by negative confounding (Choi et al. 2014) i.e., those exposed to highest levels of 2,4-D are likely to consume more fruits and vegetables, which may out weight the potential negative effect of 2,4-D

The tendencies observed suggest that *in utero* exposure to the pesticides may be associated with slightly altered androgen action during the early stages of development of the reproductive system, which may be of concern due to the widespread exposure to these pesticides and increasing use of pyrethroids.

In rodents, and probably also in humans, AGD reflect the exposure level to androgens in early development. Males have longer AGD than females and AGD is routinely used in animal toxicology studies as a sensitive marker of anti-androgenic exposures in males (Christiansen et al. 2009). High gestational exposure to testosterone has also been associated with longer AGD in female offspring in rats, suggesting a masculinization effect (Hotchkiss et al. 2007). Thus, our findings suggest that in utero exposure to these pesticides may be associated with slightly altered androgen action during the early stages of development of the reproductive system.

To our knowledge, no previous human studies have investigated associations between *in utero* exposure to pesticides and AGD in the offspring. Based on the Greenhouse Cohort children, we have previously reported higher prevalence of cryptorchidism and smaller genitals at 3 months and school age in boys whose mother were occupationally exposed to pesticides in early pregnancy compared to sons of unexposed mothers (Andersen et al. 2008; Wohlfahrt-Veje et al. 2012a). In prenatally exposed girls from the same cohort, we found earlier breast development and higher childhood serum concentrations of androstenedione and lower concentrations of Anti-Müllerian Hormone (AMH) compared to unexposed girls (Wohlfahrt-Veje et al. 2012b). Unfortunately we did not measure AGD in these children and the study design did not allow identification of specific pesticides associated with the findings.

The tendency to a dose-related elongation of female AGD associated with maternal urinary concentrations of 3-PBA and DAPs might be explained by a sexually dimorphic effect on the hypothalamic-pituitary-gonadal axis. Organophosphates and pyrethroids are neurotoxicants that may disturb development of neuroendocrine axes (Dickerson and Gore 2007) and chlorpyrifos has been reported to affect hypothalamic gonadotropin-releasing hormone (GnRH) neurons by increasing GnRH mRNA levels in female rats after *in utero* exposure and to cause earlier timing of vaginal opening and first diestrus (Gore 2001). An exposure associated increase in gonadotropins and a decline in testosterone was also reported in male rats

exposed to the pyrethroid fenvalerate (Mani et al. 2002). In humans, urinary DAP and 3-PBA concentrations were associated with increased serum concentrations of FSH, LH and prolactin and with decreased serum testosterone and inhibin B (Aguilar-Garduno et al. 2013; Meeker et al. 2009).

In mice, exposure to chlorpyrifos-methyl (4, 20 or 100 mg/kg bw/day by oral gavage) from gestational day (GD) 7 to 17 caused increased AGD in female (from 20 mg/kg bw/day) and decreased AGD in male (from 100 mg/kg bw/day) offspring (Shin et al. 2015). In rats, oral exposure to deltamethrin (1 mg/kg bw/day) from GD 7 and continued throughout the gestation and lactation was found to cause shorter AGD in male offspring (Kilian et al. 2007) while another rat study, in which the exposure (oral doses between 0.1 and 10 mg/kg bw/day) period was restricted to the period of sexual differentiation between GD 13 to 19, did not find any effect on AGD in male offspring or on expression of genes involved in the steroid synthesis pathway in the testes of male foetuses (Saillenfait et al. 2016b). Potential effects on female offspring were not included in these studies. For comparison the NOAELs set by EFSA from experimental studies included in the risk assessment was 0.1 mg/kg/day for chlorpyrifos and 1.0 mg/kg/day for chlorpyrifos-methyl, and deltamethrin. The critical effect was inhibition of Acetyl-cholinesterase or neurotoxicity in adult animals (rodents and/or dogs) whereas developmental neurotoxicity was not included. Thus, the alterations in AGD seen at NOAEL for deltamethrin or slightly above for chlorpyrifos-methyl in the studies cited above could be speculated to be related to developmental neurotoxic effects affecting hypothalamic function.

Only limited data are available on the potential of organophosphates, and pyrethroids to directly interact with sex hormone receptors and steroidogenesis. A recent review on *in vitro* studies of pyrethroids concluded that the available data do not provide evidence for strong interactions with estrogenic and androgenic pathways (Saillenfait et al. 2016a). Some of the studies included 3-PBA and other pyrethroid metabolites and found similar or greater potency than for the parent compounds (Saillenfait et al. 2016a; Tyler et al. 2000) indicating that transformation to e.g., 3-PBA in the diet or by maternal metabolizing enzymes will not always cause inactivation of potential hormone disrupting properties. Chlorpyrifos and piperophos were reported to have anti-androgenic properties and to inhibit testosterone biosynthesis *in vitro* (Viswanath et al. 2010) whereas another study, found no anti-androgenic activity for chlorpyrifos but a weak oestrogenic response (Andersen et al. 2002).

Also for 2,4-D, studies on reproductive development or sex hormone disrupting effects are sparse. No alterations in AGD were reported in rat offspring exposed to 2,4-D *in utero* and during lactation in a study performed by the manufacture Dow Chemical Company (Marty et al. 2013). No other studies investigating AGD after 2,4-D exposure were found. 2,4-D did not interact with oestrogen or androgen receptors or steroidogenesis pathways *in vitro* (Coady et al. 2014; Sun et al. 2012) but the herbicide was reported to potentiate the activity of testosterone (Sun et al. 2012) and dihydrotestosterone (Kim et al. 2005) through the androgen receptor. Enhanced androgenic activity would, however, be expected to increase AGD in males in contrast to the shorter AGD observed for the boys in our study. Thus, the lack of experimental data supporting our finding of shorter male AGD related to 2,4-D exposure, together with the lack of dose-response relationship, further indicate that this finding is likely due to chance.

Overall, the evidence for a direct interference with sex hormone receptors or steroidogenesis at relevant dose-levels for the pesticides included in this study is limited. Thus, the observed tendencies to altered AGD in girls with increasing maternal urinary 3-PBA and ∑DE concentrations are more likely related to developmental neurotoxic disturbance of the hypothalamic-pituitary-gonadal axis or they may be chance findings.

In human males, shorter AGD has been related to lower serum testosterone concentrations and symptoms of testicular dysgenesis syndrome (TDS) (Skakkebaek et al. 2016). TDS suggests a common in utero origin for several adverse male reproductive outcomes including malformations of the male genitals at birth, as well as reproductive disorders such as poor semen quality, reduced fertility or infertility, and an increased risk of testis cancer. Less is known about AGD in females and potential relation to female reproductive disorders although animal studies support an association (Crain et al. 2008; Woodruff and Walker 2008). In young adult women, AGD was reported to be positively associated with the number of follicles (Mendiola et al. 2012) and with serum testosterone concentrations (Mira-Escolano et al. 2014).

Although the associations between female AGD and maternal urinary concentrations of 3-PBA and ΣDE were non-significant and might be due to chance, the findings may be of concern due to the widespread exposure of the general population to these pesticides and the increasing use of especially pyrethroids. The effects may be related to developmental neurotoxicity of these pesticides and potential disturbance of hypothalamic development and related impact on neuroendocrine axes re (Gore et al. 2011). Such a mechanism is also supported by our findings in the Greenhouse Cohort children of disturbed reproductive development (Andersen et al. 2008; Wohlfahrt-Veje et al. 2012a; Wohlfahrt-Veje et al. 2012b) and metabolic disturbances (Tinggaard et al. 2016; Wohlfahrt-Veje et al. 2011) indicating disturbed development related to neuroendocrine signal pathways. Thus the findings deserve to be further investigated, e.g., by using data on reproductive development (including AGD) and growth patterns collected at later follow-up examinations of the children in the OCC.

5. Conclusion and perspectives

Based on the Greenhouse Cohort, we have previously found that children with an R-allele in the PON1 192 position were more susceptible towards maternal occupational pesticide exposure. Exposed children with the R-allele accumulated more body fat during childhood, had altered fat distribution, higher blood pressure and other indications of an adverse cardiovascular risk profile compared to exposed children with the RR-genotype and unexposed children. We hypothesized that the increased susceptibility were related to differential epigenetic modifications in exposed children carrying the PON1 R-allele. The results from this study support our hypothesis, as we found differentially methylated genes in several neuroendocrine signal pathways related to obesity and cardiovascular diseases. The findings deserve further investigation in a larger study with quantitative data on pesticide exposure. Since approximately 40% of the population has the PON1 192R-allele it would also be highly relevant to investigate if this genotype plays a role for pesticide related health risk at exposure levels occurring in the general population. The OCC would be an obvious opportunity for such a study.

We found widespread exposure to organophosphate and pyrethroid insecticides and to the chlorophenoxy herbicide 2,4-D with detectable metabolite concentrations in urine samples from more than 90% of the women and children in this study. The exposure levels for both organophosphates and pyrethroids were similar or higher among the children in this study than in most other published studies. This is of concern since some recent studies have reported associations between urinary concentrations of 3-PBA in children and impaired cognitive functions and behavioural problems at exposure levels below those found among the children in this study. In this study we did not find any associations between the children's current urinary 3-PBA concentrations and motor speed function or attention but studies including test that covers different cognitive domains and neurobehavioral outcomes are warranted.

The findings of an association between the children's urinary concentrations of organophosphate metabolites (DAPs and TCPY) and hit errors in the attention test among the girls may be of concern since some experimental studies have indicated that females might be more susceptible to neurotoxic effects of organophosphates than boys. Furthermore, we found girls in this cohort to be more affected by maternal occupational pesticide exposure during pregnancy than the boys. Further investigation in a larger cohort including a broader battery of tests would be highly relevant.

Among 857 mother-child pairs from the OCC we found no consistent statistically significant dose-related associations between maternal urinary concentrations of pesticide metabolites and birth outcomes or AGD in the offspring. However, the tendency towards a dose-related elongation of AGD in girls related to 3-PBA and ∑DAP (especially ∑DE) deserves to be further investigated since the exposure to pyrethroid and organophosphate insecticides is widespread and even weak effects might add to the combined effect of environmental endocrine disruptors on reproductive development.

The increasing use of pyrethroids both in agriculture and as biocides in residential settings combined with the findings in this study of a relatively high exposure level compared to other studies illustrate the need for exposure monitoring as well as more studies on potential health effects of pyrethroids. Some population studies indicate adverse effects on neurodevelopment and behavioural difficulties at lower exposure levels than seen in this study. Our findings of a

potential weak disturbance of reproductive development in females may suggest a sex-dimorphic disturbed hypothalamic function but confirmation of such a mechanism need further studies. Both pyrethroids and organophosphate insecticides are well known neurotoxicants and developmental neurotoxicity is likely to be the most sensitive effect. However, only few studies have investigated neurodevelopmental outcomes at exposure levels occurring in the general population and especially longitudinal population studies would be highly relevant. In addition, more experimental studies on potential mechanisms would be valuable. So far, developmental neurotoxicity testing has not been required for regulatory approval of pesticides or for setting of Maximal Residue Limits (MRL) in commodities. Inclusion of developmental neurotoxicity would most likely reduce the NOAEL value for most pesticides with neurotoxic properties leading to lower ADI values and MRLs and thus offer better protection of vulnerable population groups such as pregnant women and children. However, the human nervous system is very complex and not all functions can be adequately investigated in animal studies. Besides, concurrent exposure to various pesticides with similar mode of action (e.g., more different pyrethroids) might cause an effect even if the limit values for the individual pesticides are not exceeded. Therefor, population studies using validated sensitive neurodevelopmental outcomes and valid exposure information (e.g., bio-monitoring) are needed to control safe population exposure levels.

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Appendix 1.

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RESEARCH Open Access

Interaction between prenatal pesticide exposure and a common polymorphism in the *PON1* gene on DNA methylation in genes associated with cardio-metabolic disease risk—an exploratory study

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Abstract

Background: Prenatal environmental conditions may influence disease risk in later life. We previously found a gene-environment interaction between the paraoxonase 1 (*PON1*) Q192R genotype and prenatal pesticide exposure leading to an adverse cardio-metabolic risk profile at school age. However, the molecular mechanisms involved have not yet been resolved. It was hypothesized that epigenetics might be involved. The aim of the present study was therefore to investigate whether DNA methylation patterns in blood cells were related to prenatal pesticide exposure level, *PON1* Q192R genotype, and associated metabolic effects observed in the children.

Methods: Whole blood DNA methylation patterns in 48 children (6–11 years of age), whose mothers were occupationally unexposed or exposed to pesticides early in pregnancy, were determined by Illumina 450 K methylation arrays.

Results: A specific methylation profile was observed in prenatally pesticide exposed children carrying the *PON1* 192R-allele. Differentially methylated genes were enriched in several neuroendocrine signaling pathways including dopamine-DARPP32 feedback (appetite, reward pathways), corticotrophin releasing hormone signaling, nNOS, neuregulin signaling, mTOR signaling, and type II diabetes mellitus signaling. Furthermore, we were able to identify possible candidate genes which mediated the associations between pesticide exposure and increased leptin level, body fat percentage, and difference in BMI *Z* score between birth and school age.

Conclusions: DNA methylation may be an underlying mechanism explaining an adverse cardio-metabolic health profile in children carrying the *PON1* 192R-allele and prenatally exposed to pesticides.

Keywords: DNA methylation, Prenatal pesticide exposure, Paraoxonase 1, *PON1* Q192R genotype, Illumina 450 K methylation array, Cardio-metabolic health

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Background

A considerable part of modern pesticides has neurotoxic and/or endocrine disrupting properties [1–3] and therefore the potential to disturb development of neurobehavioral, neuroendocrine, and reproductive functions [4–8] especially if exposure occurs during vulnerable time periods in fetal life or early childhood. To investigate potential health effects of prenatal pesticide exposure, we have followed a cohort of children, whose mothers were employed in greenhouse horticulture in pregnancy. Some of the mothers were occupationally exposed to mixtures of pesticides in the first trimester before the pregnancy was recognized, and preventive measures were taken. Findings from this cohort include associations between maternal pesticide exposure and lower birth weight followed by increased body fat accumulation during childhood [9], impaired reproductive development in boys [10, 11], and earlier breast development [12] and impaired neurobehavioral function in girls [13].

The HDL-associated enzyme paraoxonase 1 (PON1) catalyzes the hydrolysis of a wide range of substrates including some organophosphate insecticides [14, 15]. It also protects lipoproteins from oxidative modifications and hence against development of atherosclerosis [16, 17]. A common polymorphism in the coding sequence of the *PON1* gene substitutes glutamine (Q) to arginine (R) at position 192. This substitution seems to affect both properties of the enzyme, and several studies have indicated an increased risk of cardiovascular disease in R-allele carriers [17, 18]. To investigate if this polymorphism affected the sensitivity to prenatal pesticide exposure, the *PON1* Q192R genotype was determined in the children. We found a marked interaction between prenatal pesticide exposure and the *PON1* Q192R genotype. At school age, exposed children with the R-allele had significantly higher BMI, body fat percentage, abdominal circumference, and blood pressure compared to unexposed children with the same genotype. In the group of children with the QQ genotype, there was no effect of prenatal pesticide exposure on these parameters [19]. In addition, serum concentrations of leptin, glucagon, and plasminogen activator inhibitor type-1 (PAI-1) were enhanced in prenatally pesticide exposed children with the R-allele, also after adjusting for BMI [20] which also indicates disturbance of metabolic pathways related to development of metabolic syndrome [21–23]. In addition, leptin seemed to be a mediator of the increased fat accumulation during childhood related to prenatal pesticide exposure in children with the *PON1* 192R-allele [20]. Thus, the obtained results indicate a gene-environment interaction between pesticide exposure and *PON1* gene heterogeneities already in early prenatal life that might enhance the risk of cardio-metabolic diseases later in life.

The mechanism behind this interaction is not yet understood but might be mediated by epigenetic alterations depending on both genotype and prenatal exposure. Epigenetic marks, including DNA methylation and covalent histone modifications, are dynamic and can adapt to a variety of external stimuli [24]. Furthermore, during fetal development extensive de- and re-methylation events are taking place making this period highly vulnerable for epigenetic changes caused by environmental conditions [25]. Indeed, emerging evidence in experimental animals and in humans associate altered DNA methylation patterns with a variety of prenatal exposures including dietary factors, parental care, infections, smoking, and environmental pollutants [26–31]. In experimental animals, early life changes in DNA methylation have been associated with diet-induced obesity and insulin resistance [32]. Recently, also human studies have suggested that DNA methylation patterns at birth are related to birth weight and fat mass later in childhood [33, 34]. The aim of this exploratory study was to investigate whether methylation patterns in blood samples of school children were related to prenatal pesticide exposure, *PON1* Q192R genotype, and adverse health outcomes already observed in the children. We hypothesized that the health effects associated with early prenatal pesticide exposure were related to differential epigenetic modifications in children with the QQ-genotype and children carrying the R-allele.

Methods

Study population

This study is a part of an ongoing prospective study including 203 children born between 1996 and 2001 by female greenhouse workers. The children were examined for the first time at 3 months of age [11] and followed-up at school age when 44 new age-matched controls were included [9], and the *PON1* genotype was determined for 141 children [19]. For this exploratory study, 48 pre-pubertal (Tanner Stage 1) children, whose mothers reported not to have smoked during pregnancy, were selected equally distributed between the *PON1* 192QQ and QR/RR genotype. The QR/RR genotype group consisted of 3 children with the RR genotype and 21 with the QR genotype. After excluding children of mothers who smoked in pregnancy, the number of unexposed controls within each genotype was low, 20 with the QQ genotype and 16 with the QR/RR genotype. DNA qualified for methylation analysis was only available for 11 and 12 of these children, respectively. For each genotype, we then used individual matching to select one exposed child of same sex and age for each of the controls. For the QQ-genotype, two exposed children were selected for each of two controls to obtain 24 children. Thus, in total we used data from 13 exposed and 11 unexposed children with the QQ genotype, and 12 exposed and 12 unexposed children with the QR/RR genotype (Table 1).

Table 1 Population characteristics and anthropometric data for 48 pre-pubertal children examined at age 6–11 years stratified by *PON1* Q192R genotype and prenatal pesticide exposure

	PON1 192QQ		*PON1* QR/RR	
	Unexposed	Exposed	Unexposed	Exposed
N	11	13	12	12
Female sex	5 (45.5)	7 (53.8)	6 (50.0)	6 (50.0)
Maternal smoking in pregnancy	0 (0)	0 (0)	0 (0)	0 (0)
SES[a]	7/4 (63.6/36.4)	3/10 (23.1/76.9)*	5/7 (41.7/58.3)	2/10 (16.7/83.3)
Birth weight (g)	3640 (2600; 5412)	3382 (2750; 4573)	3789 (2984; 4346)	3500 (2900; 3914)*
Gestational age (days)	276 (257; 291)	283 (265; 295)	283 (261; 298)	281 (266; 291)
Age (years)	7.6 (6.2; 9.8)	8.4 (6.7; 10.0)	7.8 (6.6; 9.5)	7.7 (7.1; 9.4)
Height (cm)	133.3 (117.3; 145.2)	130.3 (109.7; 139.2)	130.9 (113.7; 149.1)	128.6 (119.3; 142.5)
Weight (kg)	30.9 (18.7; 38.0)	28.3 (18.0; 30.7)	26.3 (19.9; 36.5)	27.4 (19.5; 37.8)
BMI (kg/m^2)	16.2 (13.7; 20.5)	15.3 (14.9; 18.3)	15.5 (13.8; 16.9)	15.7 (13.8; 19.7)
BMI *Z* scores	0.66 (−1.03; 3.21)	−0.18 (−0.80; 1.49)	−0.04 (−1.31; 0.89)	−0.01 (−0.98; 3.14)
Delta BMI *Z* score since birth	−0.45 (−2.15; 2.97)	−0.71 (−2.57; 1.87)	−0.56 (−2.52; 1.08)	0.95 (−2.08; 2.97)*
Abdominal circumference (cm)	60.4 (52.0; 75.8)	58.7 (52.1; 66.8)	58.3 (52.0; 68.1)	60.8 (51.8; 70.6)*
Sum of four skin folds (mm)	38.4 (27.1; 85.4)	33.6 (25.4; 54.5)	34.0 (20.2; 45.2)	44.6 (28.8; 72.0)*
Systolic blood pressure (mmHg)	98.7 (93.7; 110.4)	97.2 (84.3; 105.3)	99.7 (84.7; 106.8)	101.7 (91.0; 108.6)
Diastolic blood pressure (mmHg)	54.7 (46.0; 69.9)	56.2 (46.0; 62.0)	56.3 (49.3; 69.1)	63.0 (57.3; 73.1)**
Leptin (ng/ml)	1.47 (0.70; 9.18)	4.40 (0.60; 15.29)	1.41 (0.67; 5.90)	4.69 (1.79; 12.25)**
Insulin (ng/ml)	0.36 (0.22; 1.15)	0.52 (0.23; 2.55)	0.34 (0.16; 1.62)	1.11 (0.24; 7.10)*
Paraoxonase activity (nmol/min/ml)	27.5 (9.9; 38.0)	30.9 (21.0; 38.9)	58.6 (41.9; 68.7)	59.6 (50.3; 71.5)

[a]*SES* socioeconomic status (social class 1–3/4–5). Differences between unexposed and exposed children for each *PON1* Q192R genotype were tested using Mann-Whitney *U* test for continuous variables and Fisher's exact test (dichotomous variables) or Likelihood ratio (categorical variables with > 2 categories).
*P value ≤ 0.05, **P value ≤ 0.01. Values are presented as median (5–95%) for continuous variables and as *N* (%) for categorical variables

Recruitment, characteristics, exposure categorization, and clinical examinations of the children have previously been described in detail [9, 11, 19]. Briefly, we recruited pregnant women working in greenhouses and referred to the local Department of Occupational Health for risk assessment of their working conditions and guidance for safe work practices during pregnancy. Detailed information about working conditions inclusive pesticide use for the previous 3 months was obtained from maternal interview at enrollment (gestational weeks 4–10) and supplemented by telephone contact to the employers. For all women, re-entry activities (such as moving or packing potted plants or nipping cuttings) constituted their main work functions. Approximately 20% of the women reported having been directly involved in applying pesticides, mainly by irrigating fungicides or growth retardants. Only few (6%) of the women had applied insecticides. The women were categorized as occupationally exposed if pesticides were applied in the working area more than once a month, and the women handled treated plants within 1 week after treatment and/or the women were directly involved in applying pesticides. The women were categorized as occupationally unexposed if none of the above criteria was fulfilled. All exposure assessments and categorization of the mothers as pesticide exposed or unexposed were performed independently by two toxicologists before the first examination of the children. Women categorized as pesticide exposed went on paid leave or were moved to work functions with less or no pesticide exposure shortly after enrollment. Hence, the exposure classification relates to the early weeks of the first trimester before study enrollment.

The exposure situation was complex since the use of specific pesticides varied with time and location, both within the same company and between companies, depending on the plant production and the type of pest to be controlled. Out of 124 different active pesticide ingredients used in the greenhouses were 59 insecticides (17 organophosphates, 12 pyrethroids, 9 carbamates, and 21 others), 40 fungicides, 11 growth regulators, and 14 herbicides. Some were used only in few greenhouses or in short periods, whereas others were used more often. Organophosphate insecticides were used to some extent in the working areas for 91% of the exposed mothers in the entire cohort, and for 24 out of the 25 exposed mothers whose children were included in this study. The most used organophosphates were dichlorvos, dimethoate, and chlorpyrifos. Other frequently used pesticides

were the pyrethroid insecticides deltamethrin and fenpropathrin; the carbamate insecticides methiocarb, pirimicarb, and methomyl, and the fungicides fenarimol, prochloraz, tolclofos-methyl, vinclozolin, iprodion, and chlorothalonil. In general, the time interval between applying insecticides and working in the treated areas was longer (1–3 days) than for fungicides and growth regulators (often a few hours). Because of the complexity of the exposure situation and because most of the women at enrollment had been off work for some days while the risk assessment of their working conditions was performed, biomonitoring of the exposure was not feasible. A complete list of the pesticides used in the greenhouses can be obtained from the corresponding author.

At follow-up at age 6 to 11 years, 177 children underwent a standardized clinical examination in which systolic and diastolic blood pressure, pubertal staging, height, weight, thickness of skin folds, and other anthropometric parameters were measured [9]. The same pediatrician performed all clinical examinations blinded to information about maternal pesticide exposure during pregnancy.

Venous non-fasting blood samples were collected (between midmorning and late afternoon) in EDTA-coated and uncoated vials (Venoject). After centrifugation at 2000*g* for 10 min at 20 °C, buffy coat for genotyping and epigenetic analysis was separated from the EDTA-treated samples. Buffy coat and serum from the uncoated vials were stored at −80 °C until analysis.

As previously described [19], C-108T (rs705379) and Q192R (rs662) polymorphisms of the *PON1* gene was determined by the Taqman-based allele discrimination using the ABI Prism 7700 Sequence Detection System, serum activity of *PON1* was determined by spectrophotometry with paraoxon as substrate, and insulin (proinsulin and insulin) and leptin concentrations in serum were determined by commercial ELISA hormone kits from RayBio.

Genotyping and all serum analyses were performed blinded to both exposure information and examination outcomes.

Sample preparation

DNA from buffy coat samples was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The blood spin protocol was applied according to manufacturer's instructions. Samples were eluted in 100 μl elution buffer. DNA samples were bisulfite-converted using the EZ DNA methylation kit from Zymo according to manufacturer's instructions. Successful bisulfite conversion was checked using a bisulfite-specific PCR of an amplicon in the *SALL3* gene (see Additional file 1 for primer sequences). Only samples showing an intense band on agarose gel were further analyzed by the 450 K methylation array. As a negative control non-converted gDNA was used.

DNA methylation and data preprocessing

The Infinium HumanMethylation450 BeadChip array (Illumina, San Diego, CA, USA) was used to measure DNA methylation genome-wide. 4 μL of bisulfite-converted DNA from each sample was amplified, fragmented, precipitated, resuspended, and subsequently hybridized onto the BeadChips. After overnight incubation of the BeadChips, unhybridized fragments were washed away, while hybridized fragments were extended using fluorescent nucleotide bases. Finally, the BeadChips were scanned using the Illumina iScan system to obtain raw methylation intensities for each probe.

We used the R package RnBeads to preprocess the Illumina 450 K methylation data [35]. Cg-probes were filtered before normalization based on following criteria: probes containing a SNP within 3 bp of the analyzed CpG site, bad quality probes based on an iterative Greedycut algorithm where a detection *p* value of 0.01 was set as a threshold for an unreliable measurement, and probes with missing values in at least one sample. After filtering these cg-probes, beta values (ratio of methylated probe intensity versus total probe intensity) were within-array normalized using the beta mixture quantile dilation (BMIQ) method [36]. Another filtering step was performed after normalization based on the following criteria: probes measuring methylation not at CpG sites and probes on sex chromosomes. The two filtering steps removed a total of 20,338 cg-probes and ended up with a data set of normalized methylation values for 465,239 cg-probes. Beta values were transformed to *M* values ($M = \log_2(\beta/(1-\beta))$) prior to further analyses. Principal component analysis (PCA) was conducted to detect possible batch effects. Associations between the first eight principal components and possible batch effect covariates were measured. The Kruskall-Wallis test was used to find associations with sentrix_ID (BeadChip), while the two-sided Wilcoxon sum rank test was used for associations with the processing date, exposure and *PON1* Q192R genotype. Significant associations between principal component 2 and sentrix_ID (BeadChip) and processing date were suggestive for batch effects and were therefore corrected using the ComBat function in the SVA R package [37] (Additional files 2 and 3). Raw and normalized array data were uploaded to the Gene Expression Omnibus (GEO) database and have accession number: GSE90177.

For each sample, the relative cell type contribution was measured using the approach described by Houseman et al. [38]. Reference methylomes of each blood cell type (granulocyte, CD4+ T-cell, CD8+ T-cell, B-cell, monocyte, NK-cell) were obtained from the study of

Reinius et al. using the FlowSorted.Blood.450 K R package [39]. The analysis was limited to the 100,000 most variable sites. The top 500 cg-probes associated with the cell types were used to estimate the relative cell type composition in each sample. One-way ANOVA was used to determine differences in relative cell type composition between the exposed and the unexposed children and between children with the QQ and QR/RR genotype. Associations between relative cell type composition and health outcomes (percentage body fat, delta BMI z-scores from birth to school age, and BMI *Z* scores), leptin levels and age were analyzed using simple linear regression.

Statistical analysis

Differential methylation was analyzed both at the single CpG site level and at the region level (Fig. 1). At the single CpG site level, multiple linear regression (Matlab version 2014b, The Mathworks®, Natick, MA, USA) was performed in which methylation was the dependent variable and *PON1* Q192R genotype and prenatal pesticide exposure (yes/no) were the independent variables. Our statistical approach was designed to explain—at the level of methylation—the previously reported gene-environment interaction between the paraoxonase 1 (*PON1*) Q192R genotype and prenatal pesticide exposure leading to an adverse cardio-metabolic risk profile at school age among children carrying the R-allele [19]. Thus, our primary interest was to identify methylation marks associated with exposure that were more altered in R-allele carriers than in QQ-homozygotes. Two statistical models were included in our statistical approach. In the first model, effect modification (interaction) of exposure by *PON1* Q192R genotype was allowed by including main effects (exposure and genotype) and cross-product terms (exposure*genotype) in the models. Statistical significant effects of exposure in the *PON1* 192QR/RR group were defined as follows: *P* value interaction term ≤0.1 and *P* value of exposure in the QR/RR group ≤ 0.001. This model allows studying synergistic effects where the combined effect of prenatal exposure and in the QR/RR group is greater than the sum of the effects of each factor alone. In the second model, effect modification of exposure by *PON1* Q192R genotype was not assumed (no cross product term included). Statistical significant effects of exposure were defined as follows: *P* value of exposure ≤0.001, *P* value of *PON1* genotype ≤ 0.1. In this model the combined effect of exposure and being R-allele carrier is equal to the sum of the effect of each factor separately. For both models, the associations were adjusted for child sex. To identify probes that were most aberrant in the exposed QR/RR group, we set an additional filter for both models in which we defined that the prenatally exposed QR/RR group should either be highest or lowest methylated (based on mean methylation level) as compared to the other three groups (exposed QQ, unexposed QR/RR and unexposed QQ). These sites are defined as significantly differentially methylated positions (sig-DMPs) in the remainder of this text. Sig-DMPs were annotated using the HumanMethylation450 v1.2 manifest file. The freely available EpiExplorer tool was used to add further annotation including chromatin state segmentation and histone modifications based on the UCSC hg19 browser [40]. Genomic locations of transcription factor binding sites (TFBS) were directly downloaded from the UCSC h19 genome browser. Enrichment or depletion of sig-DMPs in a particular genomic region was determined using the Fisher's exact test.

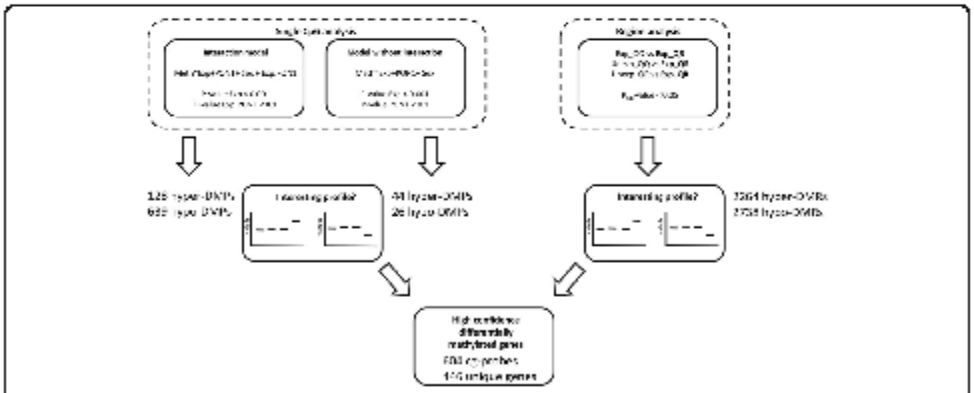

Fig. 1 Analysis workflow. Differentially methylated genes were detected using a single CpG and a region-based approach. Only sig-DMPs and sig-DMRs were selected in which the pesticide exposed QR carrier group was either hyper- or hypomethylated in comparison with the other groups (interesting profile). By overlapping the sig-DMPs with the sig-DMRs a high confidence list of differentially methylated genes could be generated

Differentially methylated regions (DMRs) were detected using the limma-based DMRcate R package [41]. We only looked for regions differentially methylated between the exposed QR/RR group and one of the other groups (exposed QQ, unexposed QR/RR and unexposed QQ). In line with identification of sig-DMPs, significant regions (P_{adj} value < 0.05) were selected in which the exposed R-allele carriers showed either the highest or the lowest methylation state which are called sig-DMRs in the remainder of this text. *P* values were corrected for multiple testing using the Benjamini-Hochberg method (P_{adj}).

Pyrosequencing

We used bisulfite pyrosequencing to further verify the methylation differences observed in the methylation array. We selected regions in four genes that are known to be involved in metabolism: *LEP*, *GPR39*, *PPARG*, and *OPCML* (Additional file 4). *LEP* DNA methylation has been associated with BMI, birth weight, and cholesterol levels [42–44]. Also, maternal conditions have an effect on the methylation status of the *LEP* promoter [45–48]. GPR39 belongs to the ghrelin receptor family and was shown to be associated with obesity [49]. PPARG is a nuclear receptor involved in regulation of lipid and metabolism as well as a target for some obesogenic endocrine disruptors [20, 50–53]. Furthermore, PPARγ is directly involved in the regulation of PON1 gene expression [54–56]. OPCML (Opioid Binding Protein/Cell Adhesion Molecule-like) is a member of the IgLON family. A SNP in the OPCML gene was associated with coronary artery calcified plaque in African Americans with type 2 diabetes [57]. A mouse and human GWAS analysis identified an OPCML SNP associated with obesity traits and visceral adipose/subcutaneous adipose ratio, respectively [58, 59]. 1 μg DNA from each sample was bisulfite-converted using the EpiTect Fast bisulfite Conversion Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. 15 ng of bisulfite-treated DNA was subsequently used in PCR amplification using the PyroMark PCR Kit (Qiagen, Hilden, Germany). Reverse primers were biotinylated to get biotin-labeled PCR products. Finally, DNA sequences were pyrosequenced using the PyroMark Q24 Advanced instrument (Qiagen, Hilden, Germany). First, streptavidin-coated Sepharose beads (High Performance, GE Healthcare, Uppsala, Sweden) were used to immobilize the biotin-labeled PCR products. Subsequently, PCR products were captured by the PyroMark vacuum Q24 workstation, washed and denaturated. The single stranded PCR products were mixed and were annealed with their corresponding sequencing primer. After the pyrosequencing run was finished, the results were analyzed using the PyroMark Q24 Advanced software (Qiagen, Hilden, Germany). Biotinylated-reverse, forward, and sequencing primers were designed using the PyroMark Assay Design 2.0 software (Qiagen, Hilden, Germany) (Additional file 1).

Mediation analysis

For a subset of sig-DMPs and sig-DMRs we analyzed (1) whether methylation is a mediator between exposure in *PON1* 192R-allele carriers and leptin levels; and (2) whether methylation is a mediator between exposure in *PON1* 192R-allele carriers and body fat accumulation (using delta BMI-score (from birth to school age), and percentage body fat as endpoints). Mediation analysis was restricted to the subset of the methylation data that overlap between the list of sig-DMPs (interaction model) and sig-DMRs. The analysis was performed by the procedure described by Baron and Kenny (1986) [60]. Leptin concentrations were logarithmically (ln) transformed prior to analysis. In mediation analysis considering body fat percentage and leptin, the models were adjusted for sex. As sex was already considered when calculating BMI *Z* score, associations considering mediation between pesticide exposure and BMI *Z* score were not adjusted for sex.

To demonstrate mediation, four requirements must be met: (model 1) the dependent outcome variable (leptin or a body fat measure) should be significantly associated with pesticide exposure (independent variable); (model 2) the DNA methylation mark (mediator) should be significantly associated with pesticide exposure; (model 3) the dependent variable should be significantly associated with the DNA methylation mark; and (model 4) the DNA methylation mark should be a significant predictor of the outcome variable, while controlling for pesticide exposure. The estimated exposure-related change in the outcome variables in model 4 should be less than in model 1 to demonstrate partial mediation, and drop to zero to demonstrate full mediation. A *P* value below 0.05 was used as a cut-off for statistical significance in each of the models.

Functionally relevant mediators, i.e., mediators that have been reported to be involved in development of weight gain/obesity, insulin resistance/diabetes, cardiovascular disease, and/or fetal growth retardation were subjected to further statistical analysis. R-package "mediation" was used to calculate the significance of the causal mediation effect using a bootstrapping approach [61]. It should be noted that the age of the children varied between 6 and 11 years at the follow-up examination where blood was collected. As child age might affect methylation levels, the exposed and unexposed children selected for this study were age-matched within each genotype.

Functional analysis

Ingenuity Pathway Analysis (IPA, Ingenuity Systems®) was used for biological interpretation. The overlap between sig-DMPs and sig-DMRs was determined and

used as input for canonical pathway analysis. A Fisher's exact test was used to determine whether the gene lists include more genes associated with a given pathway as compared to random chance (P value ≤ 0.05).

The DisGeNet platform (http://www.disgenet.org/) was used to screen for gene disease associations [62]. The database (currently) contains 429111 gene disease associations for which the platform provides a reliability score (DisGeNET Score). This score ranges from 0 to 1 and takes into account the number and type of sources (level of curation, organisms), and the number of publications supporting the association (for further details we refer to the DisGeNet website). For this manuscript, we extracted the associations with a score above 0.1. By this criterion, 34180 gene disease associations remain in the database. Associated diseases were mapped to the overlapping list of genes between sig-DMPs and sig-DMRs.

Results

Descriptive statistics of the study population

Characteristics, inclusive anthropometric data, for the 48 children (6–11 years of age) are presented in Table 1. In accordance with the findings for the whole cohort [19], birth weights were significantly lower and measures of body composition (abdominal circumference, skin fold thickness), increase in BMI Z score from birth to school age (delta BMI Z score), diastolic blood pressure, and leptin and insulin concentrations at school age were significantly higher in the exposed *PON1* 192QR/RR group compared with the unexposed QR/RR group. For children with the QQ genotype, none of the variables was significantly affected by prenatal pesticide exposure ($P > 0.05$).

Prenatal pesticide exposure-induced methylation changes at CpG sites enriched in promoter regions in *PON1* 192R-allele carriers

Genome-wide DNA methylation in whole blood samples from the children was determined by Illumina 450 K methylation arrays and differential methylation patterns related to prenatal pesticide exposure and *PON1* Q192R genotype were analyzed. First differential methylation was detected at the single CpG level using two multiple linear regression models (Fig. 1). Because relative cell type composition was not associated with pesticide exposure and *PON1* Q192R genotype (Additional file 5), differences in cellular composition were not further considered in the workflow of statistical analysis. Allowing effect modification by *PON1* Q192R genotype, 767 sig-DMPs were identified of which 128 were hypermethylated and 639 hypomethylated in prenatally exposed *PON1* 192R allele carriers. When effect modification was not assumed, and the interaction term between exposure and *PON1* genotype was removed from the models, 70 sig-DMPs of which 44 were hypermethylated and 26 hypomethylated in prenatally exposed *PON1* 192R-allele carriers were identified. Hierarchical clustering of the samples using all the sig-DMPs demonstrated a clear cluster of exposed *PON1* 192R-allele carriers (Fig. 2). Confidence in detection of differentially methylated genes was increased by further analysis showing that the changes in methylation were not restricted to single CpGs, but were often located in regions or so called differentially methylated regions (DMRs). 5002 sig-DMRs were identified, of which 2264 were hypermethylated and 2738 hypomethylated in the exposed *PON1* 192R carrier group compared to the other groups. Allowing interaction between exposure and *PON1* Q192R genotype to determine sig-DMPs, 547 out of 767 sites

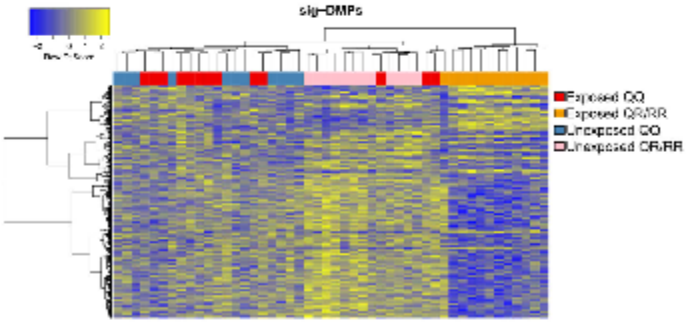

Fig. 2 Heatmap clustering representation of the sig-DMPs. Heatmap of the methylation values from the sig-DMPs showing a clear cluster of prenatal pesticide exposed *PON1*-192 R-carrier samples (*orange group*). Hierarchical clustering is based on the euclidean distance and average linkage metric. Higher methylation values are colored in *yellow*, while lower methylation values are colored in *blue*

(71.3%) were overlapping with the list of sig-DMRs (Additional file 6). When effect modification was not considered, 57 out of 70 sites (81.4%) were overlapping (Additional file 7).

The pyrosequencing methylation percentages confirmed the robustness of Illumina results. They showed significant positive correlations with the Illumina 450 K beta values for all measured CpG probes (Fig. 3), except for two probes in the *LEP* gene (cg00840332 and cg26814075) which were borderline significant (*P* value: 0.07 and 0.16, respectively). The reason for this less strong correlation between the Illumina and the pyrosequencing *LEP* methylation is probably the lower inter-individual methylation variability in this region compared to *GPR39* and *PPARG*.

In accordance with the Illumina results, the pyrosequencing *LEP* methylation values were not associated with pesticide exposure and/or *PON1* Q192R genotype. Furthermore, the serum leptin concentrations were not correlated with *LEP* methylation status (data not shown). For *GPR39*, the region analyzed with pyrosequencing contained three Illumina cg-probes (cg17172683, cg11552903, and cg18444763), which showed a high correlation ($r > 0.78$) between the Illumina beta values and the pyrosequencing methylation percentages. For most CpGs in the pyrosequencing region, we could verify a significant exposure effect, and in each CpG site, prenatally exposed children with the QR/RR genotype had the lowest mean methylation value (Additional files 8 and 9). In the *PPARG* promoter, a region was selected containing one Illumina cg-probe (cg01412654). Also here, the correlation between the 450 K Illumina beta values and the pyrosequencing methylation percentages was strong. However, DNA methylation in this region was not associated with pesticide exposure and/or *PON1* Q192R genotype and did not correlate with PON1 activity (data not shown). A region in the OPCML gene was found to be higher methylated in prenatal pesticide-exposed children carrying the *PON1* 192R-allele. The significant interaction effect between pesticide exposure and *PON1* Q192R genotype could be successfully verified by pyrosequencing. The pyrosequencing methylation values were significantly higher methylated in exposed children compared to unexposed children carrying the *PON1* 192R-allele for most of the CpG sites in the region (Additional file 9).

Next, we questioned whether the sig-DMPs were enriched or depleted in a specific genomic location (Fig. 4). Sig-DMPs for which interaction between exposure and *PON1* Q192R genotype was seen, were enriched in promoter regions (200 and 1500 bp upstream of transcription start sites) and depleted in gene bodies, 3′ UTRs and intergenic regions. This was also evident

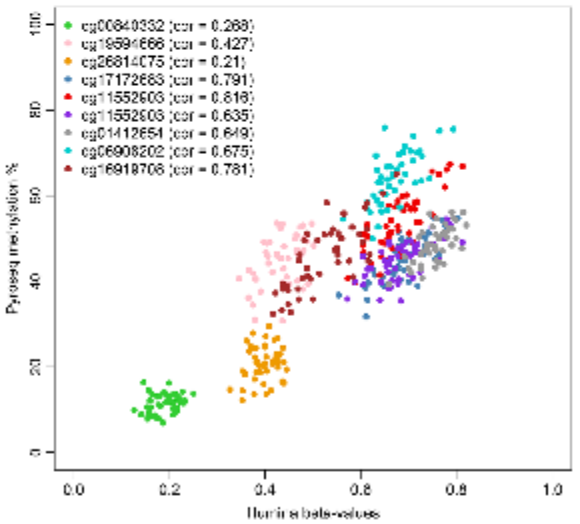

Fig. 3 Correlation between the Illumina 450 K beta values and the pyrosequencing methylation percentages. The Pearson's correlation coefficient for each CpG probe is indicated between brackets. CpG probes cg00840332, cg19594666, and cg26814075 are located in the *LEP* gene, cg17172683, cg11552903, and cg18444763 in the *GPR39* gene, cg01412654 in the *PPARG* gene, and cg06908202, and cg16919708 in the *OPCML* gene

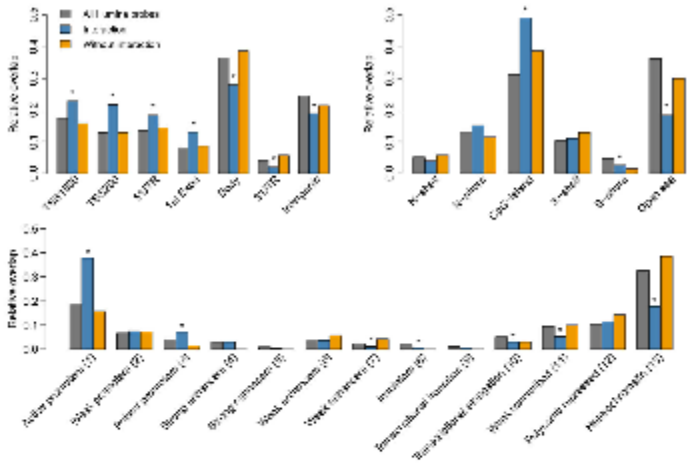

Fig. 4 Genomic location of sig-DMPs. DMPs were mapped to gene elements (*top left*), CpG islands (*top right*), and chromatin state segmentations (*bottom*). *Asterisks* indicate significant enrichment or depletion in comparison with all Illumina probes (*gray bars*) measured by the Fisher's exact test (*P* value < 0.05). Sig-DMPs where the interaction term was significant (*blue bars*) showed an enrichment in promoter regions and CpG islands. Sig-DMPs where no interaction was seen (*orange bars*) showed no significant enrichment or depletion in a particular genomic region

when we overlapped the sig-DMPs with different chromatin states, where we observed enrichment in active and poised promoters, while DMPs were depleted in regions like transcriptional elongation, weak transcribed, and heterochromatin regions. Furthermore, DMPs were significantly more located in CpG islands and less observed in CpG poor regions. Sig-DMPs found in the models without an interaction term were not enriched or depleted in a particular genomic region.

We also looked for enrichment in TFBS using available ChIP ENCODE data from the UCSC genome browser. Thirty-nine of the 161 TFBS were significantly enriched for the model with interaction (Bonferroni adjusted *P* value < 0.05) while no enrichment was found for the sig-DMPs found in the model without interaction (Additional file 10).

DNA methylation differences were enriched for genes involved in neuro-endocrine signaling pathways

Overlapping the list of sig-DMPs with the list of sig-DMRs we obtained a robust and a high confidence list of differentially methylated genes ($N = 446$). This list was used as an input for ingenuity pathway analysis. The top enriched canonical pathways (based on *P* value) were dopamine-DARPP32 feedback cAMP signaling, corticotrophin releasing hormone signaling, nNOS signaling in neurons, CDK5 signaling, and neuregulin signaling (Table 2). In the context of this manuscript, other significantly enriched pathways such as mTOR signaling (rank 9, $-\log(P \text{ value}) = 1.85$) and type II diabetes mellitus signaling (rank 16, $-\log(P \text{ value}) = 1.51$) are also highly relevant.

DNA methylation (partially) mediates associations between pesticide exposure and higher leptin concentrations, body fat content, and delta BMI *Z* scores

The list of genes that overlaps between sig-DMPs (as identified by the interaction model) and sig-DMRs was also used as input for mediation analysis. We identified, respectively, 20, 31, and 45 candidate methylation marks that (partly) mediate the effect between pesticide exposure and serum leptin concentrations; delta BMI *Z* score; and body fat content. Based on applied cut-off criteria, we were not able to identify methylation marks that mediate the effect on BMI *Z* score. Currently known gene disease associations allowed to extract mediators that were reported to be involved in development of weight gain/obesity, insulin resistance/diabetes, cardiovascular disease, and/or fetal growth retardation. This subset of mediators is given in Table 3. Based on Baron and Kenny's steps to analyze mediation, the association between pesticide exposure and delta BMI *Z* score was partially mediated by hypomethylation of *UQCRC2*, *MTNR1B* and *GRIN2A*, and by hypermethylation of *FABP4* and *LRP8*. Methylation of *UQCRC2* and *LRP8* was also a partial mediator in the association between

Table 2 Significant enriched Ingenuity canonical pathways

Rank	Ingenuity canonical pathways	−log(*P* value)	Ratio	Hyper-genes	Hypo-genes
1	Dopamine-DARPP32 Feedback in cAMP signaling	3.98	0.07	*CREB5, PPP2R2B, CACNA1A*	*KCNJ2, NOS1, GRIN2A, GUCY1B3, ADCY2, PRKCH, GNAI3, CACNA1D, PRKCG*
2	Corticotropin releasing Hormone signaling	2.74	0.07	*CREB5*	*JUND, NOS1, GUCY1B3, ADCY2, PRKCH, GNAI3, PRKCG*
3	nNOS signaling in neurons	2.61	0.11	*CAPN3*	*NOS1, GRIN2A, PRKCH, PRKCG*
4	CDK5 signaling	2.41	0.07	*PPP2R2B, CACNA1A*	*CDK5R1, NGFR, ITGA2, LAMB1, ADCY2*
5	Neuregulin signaling	2.06	0.07	*EGFR, ERBB3*	*CDK5R1, ITGA2, PRKCH, PRKCG*
6	PCP pathway	2.06	0.08		*JUND, FZD10, RSPO3, WNT7B, WNT9B*
7	Maturity onset diabetes of young (MODY) signaling	2.03	0.14	*CACNA1A*	*GAPDH, CACNA1D*
8	Regulation of eIF4 and p70S6K signaling	2.02	0.05	*PPP2R2B, FAU*	*RPS16, RPS13, RPS10, ITGA2, IRS1, RPS19*
9	mTOR signaling	1.85	0.05	*PPP2R2B, FAU*	*RPS16, RPS13, RPS10, IRS1, PRKCH, RPS19, PRKCG*
10	Amyotrophic lateral sclerosis signaling	1.84	0.06	*CAPN3, CACNA1A*	*NOS1, GRIN2A, NEFM, CACNA1D*
11	NF-κB activation by viruses	1.8	0.07		*ITGAV, CR2, ITGA2, PRKCH, PRKCG*
12	Phosphatidylethanolamine biosynthesis III	1.7	1		*PTDSS2*
13	Role of CHK proteins in cell cycle checkpoint control	1.61	0.07	*PPP2R2B, RFC4*	*E2F3, CHEK1*
14	Synaptic long-term depression	1.6	0.05	*IGF1R, PPP2R2B*	*NOS1, GUCY1B3, PRKCH, GNAI3, PRKCG*
15	ErbB signaling	1.53	0.06	*EGFR, ERBB3*	*NCK2, PRKCH, PRKCG*
16	Type II diabetes mellitus signaling	1.51	0.05	*PKM*	*NGFR, ADIPOR2, IRS1, PRKCH, PRKCG*
17	G beta gamma signaling	1.49	0.06	*EGFR*	*ADCY2, PRKCH, GNAI3, PRKCG*
18	p70S6K signaling	1.48	0.05	*EGFR, PPP2R2B*	*IRS1, PRKCH, GNAI3, PRKCG*
19	Role of osteoblasts, osteoclasts, and chondrocytes in rheumatoid arthritis	1.47	0.04		*FZD10, NGFR, SMAD5, WNT7B, ITGA2, IL1RAP, WNT9B, TCF7L2, NFATC1*
20	Molecular mechanisms of cancer	1.46	0.04		*RASGRF1, ITGA2, WNT7B, IRS1, E2F3, GNAI3, FZD10, SMAD5, ADCY2, WNT9B, PRKCH, CHEK1, PRKCG*
21	nNOS signaling in skeletal muscle cells	1.45	0.13	*CAPN3*	*NOS1*
22	Factors promoting cardiogenesis in vertebrates	1.42	0.05		*FZD10, SMAD5, PRKCH, TCF7L2, PRKCG*
23	RAR activation	1.41	0.04		*REL, ERCC2, SMAD5, NR2F1, ADCY2, PRKCH, RARB, PRKCG*
24	Choline degradation I	1.4	0.5	*CHDH*	
25	Sulfate activation for sulfonation	1.4	0.5	*PAPSS2*	
26	Mismatch repair in eukaryotes	1.4	0.13	*RFC4*	*MLH1*
27	Glioma signaling	1.37	0.05	*IGF1R, EGFR*	*PRKCH, E2F3, PRKCG*
28	Netrin signaling	1.36	0.08		*UNC5C, NCK2, NFATC1*
29	Cellular effects of sildenafil (Viagra)	1.33	0.05	*CACNG6, CACNA1A*	*KCNN1, GUCY1B3, ADCY2, CACNA1D*
30	GNRH signaling	1.33	0.05	*EGFR, CREB5*	*ADCY2, PRKCH, GNAI3, PRKCG*
31	Protein kinase A signaling	1.31	0.03	*HIST1H1A, CREB5*	*PTPN9, TIMM50, NFATC1, GNAI3, AKAP12, NGFR, PTP4A1, ADCY2, PRKCH, TCF7L2, PRKCG*
32	Ovarian cancer signaling	1.31	0.05	*EGFR*	*FZD10, WNT7B, MLH1, WNT9B, TCF7L2*
33	Colorectal cancer metastasis signaling	1.3	0.04	*EGFR*	*ADRBK1, APPL1, FZD10, WNT7B, MLH1, ADCY2, WNT9B, TCF7L2*
34	Agrin interactions at neuromuscular junction	1.3	0.06	*EGFR, ERBB3*	*ITGA2, LAMB1*
35	Growth hormone signaling	1.3	0.06	*IGF1R*	*IRS1, PRKCH, PRKCG*

pesticide exposure and body fat percentage. *LRP8* was also found to mediate the association between pesticide exposure and serum leptin concentration. The *P* value for significance of the causal mediation effect is included in Table 3 and was below 0.1 for all mediators except for *UQCRC2* and *GRIN2A*. Irrespective of disease association

Table 3 Methylation marks that partially mediate the association between pesticide exposure and leptin and body fat accumulation in *PON1*-192 R-allele carriers

Outcome	IlmnID	Nearest gene symbol	Gene name	Direction of methylation in exposed R carriers	Diseases	Significance of causal mediation effect (*p* value)
Leptin	cg03366858	*LRP8*	Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	Hyper	Myocardial infarction (0.22)|nerve degeneration (0.21)| Myocardial infarction, susceptibility to, 1 (finding) (0.2)	0.02
Leptin	cg18202502	*LRP8*	Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	Hyper	Myocardial infarction (0.22) | nerve degeneration (0.21)| myocardial infarction, susceptibility to, 1 (finding) (0.2)	0.024
Delta BMI Z score	cg00810945	*UQCRC2*	Ubiquinol-cytochrome c reductase core protein II	Hypo	Mitochondrial complex iii deficiency, nuclear type 5 (0.41) | obesity (0.21)	0.138
Delta BMI Z score	cg06337557	*MTNR1B*	Melatonin receptor 1B	Hypo	Diabetes mellitus, Type 2 (0.26)|polycystic ovary syndrome (0.21) | child development disorders, pervasive (0.21)|acute pancreatitis (0.1)	0.032
Delta BMI Z score	cg14852613	*FABP4*	Fatty acid-binding protein 4, adipocyte	Hyper	Carcinoma (0.21)|mammary neoplasms, experimental (0.21) | mammary neoplasms, animal (0.21)|insulin resistance (0.1)| erectile dysfunction (0.1)|diabetes mellitus, experimental (0.1)	0.068
Delta BMI Z score	cg15134033	*GRIN2A*	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A	Hypo	Epilepsy (0.21)|colorectal neoplasms (0.21)|epilepsy, rolandic (0.21) |melanoma (0.21)|landau-kleffner syndrome (0.21)|autistic disorder (0.21)|morphine dependence (0.21)|language development disorders (0.21)|epilepsy, focal, with speech disorder and with or without mental retardation (0.21)|speech disorders (0.21)| substance withdrawal syndrome (0.21)|rolandic epilepsy, mental retardation, and speech dyspraxia, autosomal dominant (0.2)| reperfusion injury (0.1)|hypoxia-ischemia, brain (0.1)|sepsis (0.1)| fetal growth retardation (0, 1)|central nervous system viral diseases (0.1)|placental insufficiency (0.1)	0.144
Delta BMI Z score	cg18202502	*LRP8*	Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	Hyper	Myocardial infarction (0.22)|nerve degeneration (0.21)| myocardial infarction, susceptibility to, 1 (finding) (0.2)	0.026
Bodyfat	cg00810945	*UQCRC2*	Ubiquinol-cytochrome c reductase core protein II	Hypo	Mitochondrial complex iii deficiency, nuclear type 5 (0.41) |obesity (0.21)	0.174
Bodyfat	cg03366858	*LRP8*	Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	Hyper	Myocardial infarction (0.22)|nerve degeneration (0.21)|myocardial infarction, susceptibility to, 1 (finding) (0.2)	<0.001
Bodyfat	cg18202502	*LRP8*	Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	Hyper	Myocardial infarction (0.22)|nerve degeneration (0.21)|myocardial infarction, susceptibility to, 1 (finding) (0.2)	<0.001

Only the subset of genes for which associations with metabolic disease have been reported is listed. DisGeNET Score—indicating reliability of the gene disease associations—is included between brackets

of interest, the full list of potential mediators is provided in Additional file 11 which also includes the outcome of the statistical analysis.

DNA methylation at the *PON1* promoter is affected by the *PON1*-108CT SNP (rs705379) and negatively correlated with paraoxonase 1 activity

Beside the genome-wide DNA methylation effects of the *PON1* Q192R genotype, we also observed a wide variation in DNA methylation in the *PON1* promoter itself for nine Illumina cg-probes. Prenatal pesticide exposure and/or *PON1* Q192R genotype did not affect *PON1* promoter methylation status. However, another polymorphism (rs705379, *PON1* -108CT) in the promoter region of PON1 could explain a large extent of this variation (Fig. 5). Individuals homozygous for the T-allele showed higher methylation values compared with the homozygous C-allele carriers. As expected, heterozygous individuals had an intermediate methylation value. Furthermore, the paraoxonase 1 activity was significantly associated with DNA methylation in the *PON1* promoter region, with higher methylation values resulting in lower paraoxonase 1 activity (Fig. 6). *PON1* Q192R genotype had the strongest effect on PON1 activity, while variation in PON1 promoter methylation led to a smaller but significant effect on PON1 activity.

Discussion

We found that prenatal pesticide exposure was associated with a differential DNA methylation profile in children carrying the *PON1* 192R-allele compared to children with the *PON1* 192QQ genotype and unexposed children. 767 sig-DMPs were identified of which 128 were hypermethylated and 639 hypomethylated in prenatally exposed *PON1* 192R-allele carriers. The profiles of *PON1* 192R-allele carriers are clustered together. As far as we know, our study is the first one to demonstrate a link between epigenetics and genetic susceptibility towards pesticide exposure in fetal life. Our study supports a linkage of a differential methylation pattern and higher body fat content and serum leptin concentrations in school age children dependent on both *PON1* Q192R genotype and prenatal pesticide exposure.

The majority of the detected sig-DMPs were hypomethylated in exposed children with the *PON1* 192QR/

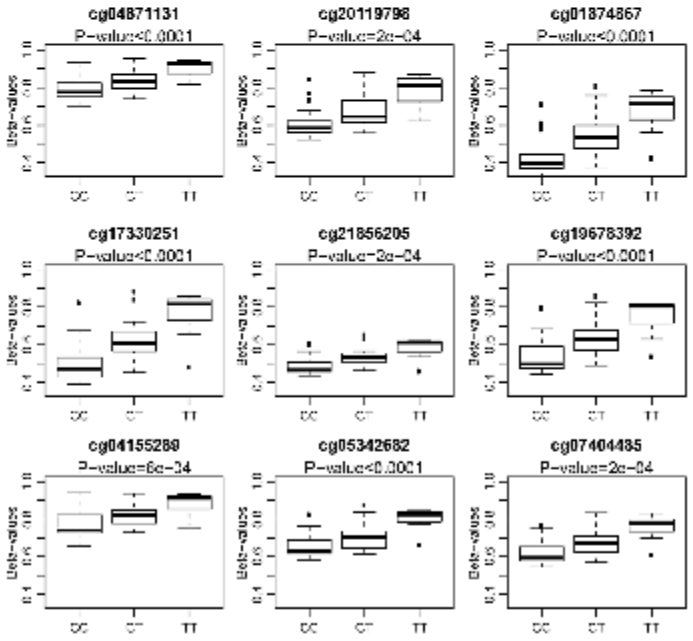

Fig. 5 Association between *PON1* methylation and *PON1* C-108T SNP. Individuals homozygous for the T allele showed higher methylation values (beta values) as compared with C-allele carriers. *P* values shown are those from the one-way ANOVA analysis

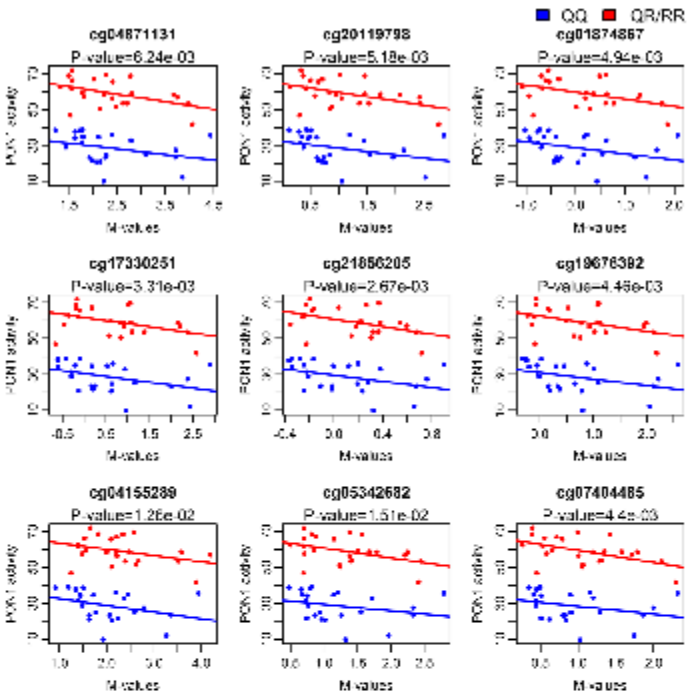

Fig. 6 Association between PON1 activity and *PON1* methylation. The *P* values of the main effect for methylation are displayed using the linear model PON1 activity ~ *M* value + *PON1*-192 genotype + sex. *Red* colored samples are *PON1* 192 R-allele carriers, and samples in *blue* are children with the *PON1* 192QQ genotype

RR genotype. Interestingly, these DMPs were mainly located in gene promoters, CpG islands and transcription factor-binding sites, suggesting a possible direct link with gene expression. To increase the confidence of our findings, we also screened for differentially methylated regions. Most of the single CpG sites were part of a DMR suggesting that these were independent of technical variation and could be considered as reliable.

Technical reliability of the outcomes from the 450 K Illumina methylation array was successfully confirmed by bisulfite pyrosequencing of corresponding CpG probe regions of four selected genes, i.e., *LEP*, *PPARG*, *GPR39*, and *OPCML* for which corresponding probes were available.

LEP was chosen because we previously found leptin to be a potential mediator of the association between prenatal pesticide exposure and body fat accumulation in children with the *PON1* 192R-allele [20]. In addition, multiple studies demonstrated associations between *LEP* DNA methylation and BMI, birth weight, and cholesterol concentrations [42–44]. *LEP* was also found to be differentially methylated in the offspring of mothers suffering from the Dutch winter famine [45]. However, our pyrosequencing results did not demonstrate a correlation between leptin DNA methylation and leptin serum concentrations, and prenatal pesticide exposure was not associated with changes in leptin DNA methylation. This suggests that the higher leptin concentration observed in exposed children with the R-allele is not due to a direct effect on DNA methylation of the leptin gene itself. Another gene whose methylation was confirmed by pyrosequencing was *PPARG*, a nuclear receptor controlling the expression of genes involved in lipid storage and glucose metabolism and target for obesogenic compounds [50–53]. Furthermore, PPARγ is involved in the regulation of *PON1* expression [54–56]. However, we did not find a correlation between *PPARG* DNA methylation and PON1 activity (data not shown). In our

dataset, prenatal pesticide exposure did not seem to change *PPARG* methylation levels irrespective of *PON1* Q192R genotype.

Reduced *GPR39* DNA methylation observed in prenatally pesticide exposed R-allele carriers was confirmed with pyrosequencing. GPR39 is receptor for obestatin (belonging to the ghrelin receptor family), involved in regulation of appetite and glucose homeostasis [63, 64] and associated with obesity [49]. Furthermore, GPR39 knockout mice showed an increased fat accumulation due to changes in lipolysis and energy expenditure [49]. So, misregulation of this gene due to methylation changes might lead to an obese phenotype. To our knowledge, no other study has yet reported methylation differences in this region associated with obesity or metabolic disorders or showed links with pesticide exposure.

The higher methylation values of the *OPCML* DMR in exposed children carrying the *PON1* 192R-allele could be confirmed by pyrosequencing. *OPCML* encodes for a protein belonging to the IgLON family. OPCML was shown to be a tumor suppressor and inactivated by DNA methylation in a variety of cancer types [65–68]. There is also a link with metabolic diseases, as SNPs in this gene were found to be associated with obesity traits, coronary artery calcified plaque, and visceral adipose/subcutaneous adipose ratio [57–59].

Further analysis revealed that the differences in DNA methylation were most pronounced in genes involved in neuro-endocrine signaling pathways, including "dopamine-DARPP32 feedback in cAMP signaling", "corticotropin releasing hormone signaling", "nNOS signaling in neurons", and "CDK5 signaling". These pathways are important in the control of food intake and energy balance. Dopamine signaling, for example, is one of the key players in the reward pathway, also controlling food intake and preferences. Reduced dopamine signaling is assumed to induce overeating [69, 70]. In mice, a high-fat diet during pregnancy resulted in altered gene expression and DNA methylation of the dopamine transporter gene in the offspring, leading to an increased preference for sucrose and fat [71]. Another study found similar results in prenatally stressed rats given a high fat-sucrose diet [72]. These studies suggest that prenatal and early life conditions may influence food intake and food preferences later in life through modulation of the dopamine pathway [73–77]. Organophosphate insecticides have been shown to modulate dopamine signaling [78]. Furthermore, low-dose exposure of neonatal rats caused metabolic dysfunction resembling prediabetes, and in adulthood, exposed animals gained excess weight when fed a high fat diet compared to unexposed rats on the same diet [79].

Corticotropin-releasing hormone (CRH) is a neuropeptide secreted in response to stress. However, a role for CRH in regulating energy balance and food intake has also been described [80–82] including a relation to the action of leptin [83].

Also NOS1 neurons are involved in energy balance and food intake [84–86]. Knock-out of *NOS1* in leptin receptor- and NOS1-expressing hypothalamic neurons results in hyperphagic obesity, decreased energy expenditure, and hyperglycemia in mice [85]. Interestingly, organophosphates have been shown to alter NOS1-expressing neurons during development in mice [87, 88].

Neureguline 1 treatment in rodents has been shown to increase serum leptin concentrations, prevent weight gain, and lower food intake. Hence, affecting this pathway may also change food intake and energy metabolism [89, 90].

A limitation of this study is that the methylation profile is measured at the same time as health outcomes and causality as such cannot be proven. Some of the genes that relate to the sig-DMPs are involved in neuroendocrine pathways that regulate appetite and energy balance, but this study cannot rule out if these sig-DMPs are a consequence of alterations of food habits and physical activity among the exposed children with the *PON1* 192R-allele or an underlying mechanism. However, the mediation analysis suggested that some of the differentially methylated marks are on the mechanistic pathway between prenatal pesticide exposure and the measured outcomes. This result suggests that, at least in some, CpG sites a change in methylation might contribute to metabolic disturbances later in life. Furthermore, the association was not significant between pesticide exposure and BMI *Z* score as such, but between pesticide exposure and delta BMI *Z* score which integrates fat accumulation from birth and onwards to school age.

Interestingly, some of the mediator marks could be linked to specific genes that were reported earlier to play a role in the development of weight gain/obesity, insulin resistance/diabetes, cardiovascular disease, and/or fetal growth retardation: *UQCRC2*, *MTNR1B*, *GRIN2A*, *FABP4*, and *LRP8*. *FABP4* encodes for a member of the fatty acid-binding protein family regulating lipid trafficking, signaling, and metabolism. Different studies have demonstrated the role of this protein in obesity, type 2 diabetes and atherosclerosis development [91–93]. In ApoE deficient mice with hyperhomocysteine *FABP4* DNA methylation is reduced in the aorta compared to wild type mice, leading to a higher gene expression [94, 95]. *UQCRC2* encodes a protein which is part of the ubiquinol-cytochrome c reductase complex in the mitochondria. UQCRC2 was shown to be downregulated in individuals who were susceptible to weight gain and obesity development [96]. The melatonin receptor 1B (MTNR1B) has a main function in regulating circadian rhythm. Interestingly, several polymorphisms in the

MTNR1B gene are associated with type 2 diabetes, fasting glucose concentration, and insulin secretion [97–99]. *GRIN2A* encodes for a NMDA glutamate receptor subunit. Polymorphisms in the *GRIN2A* gene are associated with epilepsy and different neurological and mental disorders [100–104]. A decreased gene expression of GRIN2A in rats after intrauterine growth retardation suggests a possible role for this gene in fetal growth and development [105]. *LRP8* encodes for a member of the LDL receptor family. Common polymorphisms in the *LRP8* gene are associated with coronary artery disease, myocardial infarction, and high birth weight [106–110]. Thus, the mediation analysis suggests a mechanistic role of epigenetics in the development of an adverse metabolic risk profile among the prenatally exposed children with the *PON1* R-allele as previously reported for these children [19] and confirmed in the selected subset of children.

A few studies have investigated associations between PON1 genotype and metabolic disturbances in children. A recent study showed a higher risk of insulin resistance (HOMA-IR) in Mexican children with the RR-genotype as compared to children with the QQ or QR genotypes although BMI did not differ between the groups [111]. Among Mexican-American children from an agricultural community in California, a trend of increased BMI *Z* scores with increased number of PON1 192Q alleles was seen [112]. However, potential interactions between PON1 genotype and prenatal exposure to pesticides, or other environmental contaminants, were not investigated in these studies. In our cohort, unexposed QQ-homozygote children also tended to have higher body fat content than unexposed R-carriers, but prenatally pesticide exposed children with the R-allele accumulated more fat during childhood and had a more unhealthy metabolic risk profile at school age than unexposed children and exposed children with the QQ genotype [19].

We also demonstrated that methylation in the *PON1* promoter itself is affected by a SNP (*PON1* -108CT, rs705379). In addition, *PON1* methylation values were negatively associated with paraoxonase 1 activity. These results are in agreement with the outcome of a recent study from Huen and colleagues [113]. They found methylation in the same nine CpG sites to be associated with the *PON1* -108CT polymorphism and also reported an inverse association with AREase activity as a measure of *PON1* expression, both in newborns and 9-year-old children. Furthermore, they demonstrated that *PON1* methylation mediates the relationship between *PON1* expression and the promoter -108 genotype. However, the effect of prenatal pesticide exposure on the health outcomes shown in Table 1 was not modulated by PON1 -108CT genotype (data not shown).

Our findings indicate that the higher vulnerability among children with the R-allele towards prenatal pesticide exposure might be mediated by genotype-specific epigenetic alterations. However, a limitation of this study is that we cannot identify individual pesticides related to these findings, since the study design did not allow bio-monitoring of pesticide exposure in the mothers, and the exposure classification of the mothers encompassed more than 100 pesticides used in different mixtures [11].

However, the existence of mixed exposure is a real-world situation, and the longitudinal design, the blinded exposure classification, and the blinded clinical examinations, and genotyping minimized the possible impact of exposure misclassification and bias.

Since PON1 is known to detoxify some organophosphate insecticides (e.g., chlorpyrifos), and these substances were frequently applied in the mothers' working areas, organophosphate insecticides could be assumed to be responsible for the observed effects. However, the mechanism is unclear and does not seem to be related to the hydrolysis efficiency, since R-carriers have higher paraoxonase activity than QQ homozygotes. Besides, at relatively low exposure levels, as in this study, the capacity to detoxify organophosphates is considered to be independent of the *PON1* Q192R genotype [114], and furthermore, serum PON1 activity was reported to be low in newborns and may be even lower before birth, as indicated by lower activity in premature compared to term babies [115, 116]. Thus, differences in fetal detoxification of pesticides related to *PON1* genotype might not be a likely explanation of the exposure-related difference in methylation pattern between children with the QR/RR and QQ genotype.

Another limitation of the study is that DNA methylation analyses were performed in white blood cells as surrogates for the target tissues. We do not know whether the differences in DNA methylation patterns found in blood mirror a similar change in adipose tissue, for example. A recent study from Huang et al. demonstrated several potential limitations in using methylation profiles in blood to mirror the corresponding profile in target tissues by comparing paired blood and adipose tissue methylation profiles [117]. Furthermore, the composition of blood cell types may be variable and might affect the DNA methylation analyses. In our dataset, prenatal pesticide exposure and/or *PON1* Q192R genotype did not affect the relative blood cell counts determined by the reference-based method of Houseman. Cell counts were not included in the models due to the small sample size of the study. Since we found that some of the health effects (mainly leptin) were associated with cell type count (Additional file 12), we cannot exclude that the results of the mediation analysis were biased by differences in cell type composition. Based on the data of Reinius et al. [39], methylation of only two CpG probes

(cg18202502 and cg15134033) in Table 3 were slightly associated with cell types (data not shown). Methylation in the other CpG probes in Table 3 was not significantly different between the blood cell types.

Finally, the small number of subjects included in this exploratory study is a clear weakness because of the limited statistical power. Despite these constraints, our findings suggest that DNA methylation might be a link between prenatal pesticide exposure and cardio-metabolic risk profile in children carrying the PON1 192R-allele. The findings deserve further investigation in a larger study with quantitative data on pesticide exposure. Whether this DNA methylation pattern is unique to pesticide exposure or is shared by other adverse prenatal environmental factors also needs further investigation.

Conclusions

In summary, our data indicate that DNA methylation may be an underlying mechanism explaining an adverse cardio-metabolic risk profile in prenatally pesticide-exposed children carrying the *PON1* 192R-allele.

Additional files

Additional file 1: Primer sequences. (XLSX 10 kb)

Additional file 2: PCA before and after batch effect correction for Sentrix_ID and processing date using ComBat. (TIFF 240 kb)

Additional file 3: Associations between the first eight principal components and covariates before and after ComBat batch correction. Associations between principal components and Sentrix_ID were measured using the Kruskal-Wallis test. Associations between principal components and processing date, exposure and *PON1* Q192R genotype were measured using the two-sided Wilcoxon sum rank test. (TIFF 102 kb)

Additional file 4: Genomic location of the pyrosequencing assays represented as a UCSC genome browser track. The first track indicates the sequence analyzed by pyrosequencing (Seq_to_analyse). Other custom tracks include CpG islands, DNase I hypersensitivity clusters, H3K27ac histone marks, transcription factor-binding sites, and the Illumina 450 K methylation probes. A) *LEP* assay B) *GPR39* assay C) *PPARG* assay and D) *OPCML* assay. (TIFF 3014 kb)

Additional file 5: Relative cell type contribution estimated by the Houseman approach. Differences in cell type composition between the exposure groups were measured using one-way ANOVA. (TIFF 259 kb)

Additional file 6: Sig-DMPs overlapping with DMRs (interaction model). For each DMP *P* values are given for the interaction between pesticide exposure and *PON1* genotype (P.Value.Int_EXPPON1), and for the exposure effect PON1 R-allele carrier group (P.Value.EXP_when PON1 QR/RR). The mean beta values in each exposure group are listed. (XLSX 82 kb)

Additional file 7: Sig-DMPs overlapping with DMRs (model without interaction). For each DMP *P* values are given for the *PON1* effect (P.Value.PON1) and exposure effect (P.Value.EXP). The mean beta values in each exposure group are listed. (XLSX 16 kb)

Additional file 8: Outcome of GPR39 DMR pyrosequencing. *Boxplots* showing methylation differences between the exposure groups in the *GPR39* pyrosequencing region. *P* values shown are those of the exposure effect. (TIFF 193 kb)

Additional file 9: Outcome of GPR39 DMR pyrosequencing. (XLSX 11 kb)

Additional file 10: Enrichment of TFBS for DMPs significant in the interaction model. *P* value from the Fisher's exact test were adjusted using the Bonferroni correction. (XLSX 10 kb)

Additional file 11: Mediation analysis. Outcome statistics and gene disease associations of (partial) mediators between pesticide exposure and body fat measures in *PON1* R-allele carriers. (XLSX 47 kb)

Additional file 12: Association between estimated blood cell counts and health outcomes. Simple linear regression was used to determine associations between the relative blood cell type composition and the health outcomes (body fat, BMI *Z* score, delta BMI *Z* score, leptin levels, and age). (XLSX 11 kb)

Abbreviations

DMP: Differentially methylated position; DMR: Differentially methylated region; IPA: Ingenuity Pathway Analysis; PAI-1: Plasminogen activator inhibitor type-1; PON1: Paraoxonase 1; TFBS: Transcription factor binding site

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus (GEO) repository with accession number GSExxxx.

Authors' contributions

KD, SR, GS, WVB, and HRA conceived and designed the experiments. KD, SR, and HRA performed the experiments and analyzed the data. CWV, KMM, GVC, GS, WVB, and HRA contributed reagents/materials/analysis tools. KD, SR, GS, WVB, and HRA wrote the paper. KD, SR, CWV, KMM, GVC, GS, WVB, and HRA evaluated the manuscript text. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study was conducted according to the Helsinki II Declaration with written informed consent by all parents and oral consent by the children as approved by The Regional Scientific Ethical Committees for Southern Denmark (S-20070068) and the Danish Data Protection Agency.

Publisher's Note

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Appendix 2.

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Associations of maternal exposure to organophosphate and pyrethroid insecticides and the herbicide 2,4-D with birth outcomes and anogenital distance at 3 months of age in the Odense Child Cohort

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Abstract

Many pesticides found as residues in food items possess endocrine disrupting properties. In the Odense Child Cohort (OCC), concentrations of the pesticide metabolites 3-phenoxybenzoic acid (3-PBA), 3,5,6-trichloro-2-pyridinol (TCPY), 2,4-Dichlorophenoxyacetic acid (2,4-D) and dialkyl phosphates (DAPs) were measured in urine samples collected in gestational week 28 in up to 858 pregnant women. Gestational length, birth weight, head and abdominal circumference were obtained from birth records and anogenital distance (AGD) was measured at a physical examination three months after the expected date of birth. The pesticide metabolites were detectable in most of the urinary samples (>93%). We did not find consistent statistically significant dose-related associations between pesticide metabolite concentrations and birth outcomes or AGD. However, a non-significant dose-related elongation of AGD in girls was seen for 3-PBA (AGDac, p-trend: 0.14) and diethyl phosphate (DE) metabolites (AGDac, p-trend: 0.08). These tendencies may suggest a possible weak disturbance of sex hormone action.

Keywords: endocrine disruption, reproduction, organophosphate insecticides, chlorpyrifos, pyrethroids, 2,4-D, anogenital distance

1. Introduction

Organophosphate and pyrethroid insecticides and the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) are some of the most commonly used pesticides worldwide and residues of these pesticides are among the most frequently detected in food items at the European market [1]. In Denmark, organophosphates are no longer allowed for food production but residues are still frequently detected in imported food items with residues of chlorpyrifos being most prevalent [2]. While the quantity of organophosphates used is declining, the use of pyrethroids is increasing both for agricultural use and for residential pest control [3]. 2,4-D is used to control broadleaf weeds in agricultural and residential settings and as a growth regulator in citrus fruit production [4]. All these pesticides are non-persistent with biologic half-lives of hours to days and they are able to cross the placenta in humans [5]. In experimental studies, 2,4-D and some organophosphates and pyrethroids have been identified as potential endocrine disruptors with capacity to interfere with sex hormone action [6-9].

We previously reported higher prevalence of cryptorchidism and smaller genitals at age 3 months and at school age in boys whose mothers were occupationally exposed to pesticides in early pregnancy compared to sons of unexposed mothers [10, 11]. In prenatally exposed girls from the same cohort, we found earlier breast development and higher childhood serum concentrations of androstenedione compared to unexposed girls [12]. However, potential adverse effects on reproductive development, e.g. anogenital distance in humans at exposure levels occurring in the general population have not been investigated.

Anogenital distance (AGD), the distance between anus and genitals, is routinely used in rodent studies as a marker of androgen action during an early fetal reproductive programming window [13]. AGD is sexual dimorphic with males having a larger AGD than females [14]. Insufficient androgen action reduces AGD in male rats whereas excessive androgen exposure increases AGD in female rats [13]. Human studies suggest similar adverse effects, as maternal exposure to phthalates (with anti-androgenic action) [15] has been associated with shorter AGD in male offspring [16]. Few human studies have examined AGD in females. In rodents, the pyrethroid deltamethrin [17] and the organophosphates fenitrothion [18] and chlorpyrifos-methyl [19] have been associated with altered AGD in offspring but to our knowledge no human studies have been conducted. We therefore investigated whether the maternal urinary concentrations of 2,4-D and organophosphate and pyrethroid insecticide metabolites were associated with birth outcomes (birth weight, head and abdominal circumference and gestational

length) and AGD in the offspring at three months of age in 858 mother-child pair from the Odense Child Cohort.

2. Materials and Methods

2.1 Study population

Participants for this study were derived from the Odense Child Cohort (OCC). Briefly, all women living in the municipality of Odense who were newly pregnant between 1st of January 2010 and 31st of December 2012 were invited to participate (a total of 6707). They were recruited either at a voluntary meeting regarding ultrasound examinations, at their first antenatal visit or at the ultrasound examination at Odense University Hospital between gestational age (GA) 10–16 weeks [20]. Of the eligible women, 4017 were informed and 2874 (43%) agreed to participate.

At GA 28 weeks, the participating women donated a urine sample, and twice during pregnancy, they filled out a questionnaire on e.g. general health, lifestyle and social factors. Information on maternal age, ethnicity and educational level was obtained from the questionnaire completed at the time of inclusion, while information on parity, smoking and child sex was derived from the birth records. Information on educational level was missing in the questionnaires for 132 women for which information on occupation was retrieved from the birth records and used to estimate educational level whenever possible.

2.2 Birth outcomes and AGD

Information about birth weight (grams), head circumference (cm), abdominal circumference (cm) and gestational age (days) was obtained from birth records. A clinical examination including measurements of length, weight and AGD was scheduled to take place three months after expected due date, regardless of actual gestational age at birth. The AGD was measured using a Vernier caliper and expert-trained technicians conducted the measurements. In boys, a short AGD was measured from the center of anus to the posterior base of scrotum (AGDas) and a long AGD was measured from the center of anus to the cephalad insertion of the penis (AGDap). Correspondingly in girls, a short AGD was measured from the center of anus to the posterior fourchette (AGDaf) and a long AGD from the center of anus to the top of clitoris (AGDac) The measurements were conducted three times for each child in order to decrease the risk of measurement bias. An arithmetic mean was calculated for each type of AGD measure on each child. For all three AGD measurements the coefficient of variation (CV) was below 10 %, except for AGDaf, in which two girls had CV's of 10 % and 14 %, respectively. Four trained

technicians performed all measurements in order to minimize the risk of inter-observer variations [21].

2.3 Exposure measurements

The participating women donated a urine sample around gestational week 28 (range 27.0 - 28.6) and due to other examinations being conducted the same day the women were fasting. A total of 858 women had their urine analyzed for the specific metabolite of chlorpyrifos/chlorpyrifos-methyl, TCPY (3,5,6-trichloro-2-pyridinol), the generic pyrethroid metabolite, 3-PBA (3-phenoxybenzoic acid) and the herbicide 2,4-D, whereas 564 of these also had their urine analyzed for six unspecific OP metabolites (dialkyl phosphates, DAPs): three dimethyl (DM) phosphate metabolites (dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), dimethyl dithiophosphate (DMDTP)) and three diethyl (DE) phosphate metabolites (diethyl phosphate (DEP), diethyl thiophosphate (DETP), and diethyl dithiophosphate (DEDTP)). The first 200 urine samples were selected randomly, whereas the remaining were selected based upon availability of information from questionnaires, birth records and AGD measurements from the three-month examination. The urine samples were stored in freezers at -80 degrees Celsius at the Odense Patient data Explorative Network (OPEN) [20] until analysis.

Urine concentrations (µg/L) of TCPY, 3-PBA, and 2,4-D were measured by reversed-phase high performance liquid chromatography and tandem mass spectrometry with isotope dilution quantitation, according to the method described by Davis et al. [22] after minor modifications. Spectrophotometric determination of creatinine concentrations was conducted on a Konelab 20 Clinical Chemistry Analyzer, using a commercial kit (Thermo, Vantaa, Finland). The analysis of TCPY, 3-BPA, 2,4-D and creatinine were performed at the Environmental Medicine Laboratory, University of Southern Denmark (SDU).

Urine analyses of DAPs were performed at the Flemish Institute for Technological Research NV (VITO), Belgium using solid phase extraction (SPE) followed by Ultra Performance Liquid Chromatography-tandem mass spectrometry (UPLC-MS/MS). Briefly, the urine samples were acidified and spiked with mass-labeled internal standards and concentrated using SPE. The compounds of interest were eluted with 5% NH_4OH in methanol. The extract was evaporated to dryness and reconstituted with UPLC-grade water. An aliquot of the extract was injected into the LC–MS/MS system (Waters, Milford, MA, USA). The phosphate metabolites were separated on an Acquity UPLC RP shield column (100 mm × 2.1 mm; 1.7 µm). The column tem-

perature was kept at 40 ◦C. Optimum separation was obtained with a binary mobile phase constituted of ultrapure water (solvent A) and acetonitrile (solvent B), both solvents acidified with 0.1% formic acid. The flow rate of the mobile phase was 0.4 mL/min. The UPLC system was coupled to a Waters Xevo TQ-S tandem mass spectrometer and operated in the negative electrospray ionization mode (ESI−). The system was operated in multiple reaction-monitoring (MRM) mode after selection of the characteristic precursor and product ions of each analyte. The metabolite concentrations were converted from µg per liter urine to their molar concentrations (nmoles per liter) and summed to obtain the total concentration of dimethyl phosphate metabolites ((DM), sum of DMP, DMTP, and DMDTP), diethyl phosphate metabolites ((DE), sum of DEP, DEDTP, and DEDTP), and total DAP (sum of all six metabolites).

2.2 Ethics

The study was conducted in accordance with the principles of the Declaration of Helsinki and the women provided written informed consent to participate. The study was approved by The Regional Committees on Health Research Ethics for Southern Denmark (S-20090130) and the Danish Data Protection Agency (13/14088).

2.3 Statistics

To correct for urinary dilution, all pesticide metabolite concentrations were expressed per gram creatinine.

The distributions of all urinary pesticide concentrations were not normally distributed and therefore reported as medians. For further analyses, the continuous concentrations were both transformed by the use of natural logarithm (ln(2)) and divided into tertiles. Differences in concentrations according to maternal characteristics were tested by Kruskal Wallis test or Willcoxon rank sum test.

Linear regression analysis was conducted to estimate associations between creatinine adjusted maternal urinary TCPY, 3PBA, 2,4-D, ΣDAP, ΣDE and ΣDM concentrations, and birth outcomes and AGD at three months of age and adjusted for potential confounders. Confounders included in the final models were known to be possible predictors for birth outcomes or AGD and were associated with pesticide exposure. The models investigating pesticide metabolites and birth weight, head and abdominal circumference were adjusted for maternal education, pre-pregnancy body mass index (BMI), smoking and gestational age, whereas the models of gestational age were adjusted for maternal educational level, pre-pregnancy BMI and smoking. All analyses were stratified by child sex as sex dimorphic effects were expected. In

the models for birth outcomes, the analyses with 3-PBA were repeated adjusting for parity as the concentration of 3-PBA (but none of the other metabolites), seemed to be related to the number of previous births.

AGD is associated with weight and age and a measure of “post-conceptual-age” was therefore constructed. The “post-conceptual-age” was defined as the sum of gestational age at birth (days) and the age of the child (days) at the three-months examination. Furthermore, an age-and-sex specific Z-score for the weight of the child was calculated from all 2041 singleton children who completed the three-months examination in the OCC. The linear regression models of the association between pesticide metabolites and AGD were thus adjusted for weight-for-age Z-score and age at 3-month examination. Due to known ethnic differences in size at birth and AGD [23], we repeated the analyses after excluding women with non-Western ethnicity (mother or maternal parents born in Western countries (yes/no). We also did a sub analysis adjusting for maternal education level. In all final models, we checked for normal distribution of the residuals as well as linearity, homogeneity and outliers. The model assumptions for linear regression analysis seemed satisfied. Analyses were conducted in STATA 14 and results are presented with 95% confidence intervals, and p-values <0.05 were considered statistically significant.

3. Results

The mean age of the participating women was 30.3 years, 56% of the women were primiparous, 97 % were of European origin and 4% smoked during pregnancy. Mean gestational age was 280 days whereas the mean birth weight was 3606 g for boys and 3475 g for girls. The mean abdominal circumferences at birth were 33.1 and 33.0 cm, and the mean head circumferences were 35.4 and 34.8 cm for boys and girls, respectively. Significantly fewer smokers (4% vs. 7% p=0.007) and mothers of non-European origin (3% vs. 6% p<0.001) in our study population compared to all participating women in the OCC were found. Further, pre-pregnancy BMI was higher in our study population (p=0.008). Maternal age, parity and birth measurements did not differ (data not shown).

Concentrations of 2,4-D were detectable in 97.6%, 3-PBA in 94.3% and TCPY in 93.1% of the mothers (Table 1). Mothers with a high pre-pregnancy BMI had lower concentrations of DAPs and 2,4-D while 3-PBA tended to be higher (Table 2). Further, 3-PBA was lower in multiparous women. A higher maternal education was associated with higher concentrations of TCPY and 2,4-D (Table 2).

No clear patterns of associations between maternal pesticide exposure and birth outcome were seen. Gestational length tended to be longer for boys in the third tertile of maternal DAP concentration compared to the boys in the first tertile (3.2 (95% CI: 0.1, 6.4) days, p-trend: 0.05)(Table 4). Further, a tendency towards a smaller abdominal circumference with increasing 3-PBA concentrations was seen in girls (β: -0.3 (95%CI: -0.5, -0.003) cm). Further adjustment for parity did not change the association (data not shown).

We did not find statistically significant dose-related associations between the maternal urinary concentrations of any pesticide metabolite and AGD in the offspring (Figure 1 and 2). However, a tendency towards a longer AGD with higher maternal concentrations of 3-PBA (AGDac, p-trend: 0.14) and ΣDE (AGDac, p-trend: 0.08) was seen in girls. Further, a statistically significant shorter AGD in boys with maternal concentrations of 2,4-D in the second compared to the first tertile (AGDas: -1.55 (95% CI: -2.81, -0.28) mm and AGDap: -1.62 (95% CI: -3.00, -0.24) mm) was found (Table 3). Analyses excluding women of non-Western origin or further adjustment for maternal educational level did not affect the findings.

4. Discussion

In this prospective study of 858 mother-child pairs, we did not observe any statistically significant dose-response associations between maternal urinary concentrations of pesticide metabolites and AGD in their offspring at three months of age. However, weak sexually dimorphic associations were indicated, as 3-PBA and ∑DE tended to be dose-related to a longer AGD_{ac} in girls and as boys in the intermediate tertile of 2,4-D compared to the first tertile had shorter AGDs. However, since no change was seen from the first to the third tertile in boys, the finding may be due to chance, e.g. caused by multiple comparisons.

The tendencies observed in girls suggest that *in utero* exposure to the pesticides may be associated with slightly altered androgen action during the early stages of development of the reproductive system, which may be of concern due to the widespread exposure and increasing use of pyrethroids. We did not find any consistent associations between the urinary concentrations of the pesticide metabolites and gestational length, birth weight, or head and abdominal circumference.

TCPY, 3-PBA and 2,4-D were detectable in more than 90 % of the women in our study indicating a widespread exposure of the Danish population. The urinary concentrations of DAPs, TCPY, 3-PBA, and 2,4-D were in general lower than reported in recent studies from the US

and Canada [24-28], but similar or higher than concentrations reported in most European studies [29-33]. However, the urine samples analyzed in our study were obtained after over-night fasting and this might have underestimated the exposure levels.

Occupational or residential exposure to pesticides during pregnancy has in some studies been related to lower birth weight [34, 35] or length [36] in the offspring or shorter gestational length [37]. In some of these studies, the adverse birth outcomes were demonstrated to be associated with maternal urinary concentrations of TCPY or DAPs. However, at lower exposure levels, as seen in the general population, there seem to be no consistent association to adverse birth outcomes [28, 38]. Only few studies have investigated associations between maternal urinary concentrations of 3-PBA and fetal growth indices and none of these studies reported significant adverse effects [39-41]. No studies investigating exposure to 2,4-D and birth outcomes were found. Thus, the findings from our study of no consistent associations between maternal urinary pesticide metabolite concentrations and birth outcomes are in agreement with findings from other studies based on non-occupational exposed populations with low residential use of pesticides.

To our knowledge, no previous human studies have investigated associations between *in utero* exposure to pesticides and AGD in the offspring. We previously reported higher prevalence of cryptorchidism and smaller genitals in boys and earlier breast development and higher childhood serum concentrations of androstenedione in girls whose mother were occupationally exposed to pesticides in early pregnancy compared to children of unexposed mothers [10, 11]. Unfortunately, we did not measure AGD in these children and the study design did not allow identification of specific pesticides associated with the findings.

The tendency to a dose-related elongation of female AGD associated with maternal urinary concentrations of 3-PBA and DAPs (especially DEs) seen in our study might be explained by a sexually dimorphic effect on the hypothalamic-pituitary-gonadal axis. OPs and pyrethroids are neurotoxicants that may disturb development of neuroendocrine axis [42]. Thus, chlorpyrifos has been reported to affect hypothalamic gonadotropin-releasing hormone (GnRH) neurons by increasing GnRH mRNA levels in female rats after *in utero* exposure and to cause earlier timing of vaginal opening and first diestrus [9]. Little is known about AGD in females and potential relation to female reproductive disorders although animal studies support an association [43-45]. In young adult women, AGD was reported to be positively associated with the number of

follicles [46] and with serum testosterone concentrations [47]. A possible association between prenatal exposure to the pesticides and AGD in females are thus of concern as it may affect the reproductive system in later life.

Although we did not find any clear tendencies of altered AGD in males after in utero pesticide exposure in this study, studies among adult men also indicate that OPs and pyrethroids may interfere with the hypothalamic-pituitary-gonadal axis. Among male floriculture workers, urinary DAP concentrations were associated with increased serum concentrations of FSH and prolactin and with decreased serum testosterone and inhibin B [48]. Furthermore, population representative urinary concentrations of pyrethroid metabolites, including 3-PBA (within the same range as this study), have been associated with reduced semen quality [49], higher serum concentrations of FSH and LH, and lower inhibin B, and testosterone [50]. In accordance with these findings, the pyrethroid fenvalerate caused increased gonadotropins and a decline in testosterone in male rats [51]. Further, exposure to deltamethrin throughout gestation and lactation caused shorter AGD in male offspring [17], whereas no effects on AGD or on expression of genes involved in testicular steroidogenesis in the testes was observed when the exposure period was restricted to the period of sexual differentiation between gestational day 13 and 19 [52]. None of these studies investigated female offspring. In mice, gestational exposure to chlorpyrifos-methyl caused longer AGD in female offspring at 20 mg/kg /day and shorter AGD in male at 100 mg/kg/day [19].

Only limited data are available on the potential of OPs and pyrethroids to interact with sex hormone receptors and steroidogenesis. A recent review on *in vitro* studies of pyrethroids concluded that the available data do not provide evidence for strong interactions with estrogenic and androgenic pathways [6]. Some of the studies included 3-PBA and other pyrethroid metabolites and found similar or greater potency than for the parent compounds [6, 53] indicating that transformation to e.g., 3-PBA in the diet or by maternal metabolizing enzymes might not eliminate potential endocrine disrupting effects of pyrethroids on the fetus. Chlorpyrifos and piperophos were reported to have anti-androgenic properties and to inhibit testosterone biosynthesis *in vitro* [7]. Also for 2,4-D, studies on reproductive development or sex hormone disrupting effects are sparse. No alterations in AGD were reported in rat offspring exposed to 2,4-D *in utero* and during lactation [54]. 2,4-D did not interact with estrogen or androgen receptors or steroidogenesis pathways *in vitro* [8, 55] but the herbicide was reported to potentiate the activity of testosterone through the androgen receptor [8], which would be expected to increase AGD in males in contrast to the decrease observed in our study. Overall, the evidence for a

direct interference with sex hormone receptors or steroidogenesis at relevant dose-levels for the pesticides included in this study is limited. Thus, the observed alterations in AGD are more likely related to developmental neurotoxic disturbance of the hypothalamic-pituitary-gonadal axis or they may be chance findings.

Our study has several strengths and limitations. It is population based with prospectively collected data. Birth outcomes were well reported and AGDs were measured three times by trained technicians. However, only 42% of the eligible women participated in the OCC and participants were better educated and more often of Danish origin than non-participants. However, the pregnant woman had no prior knowledge of their urinary pesticide concentrations or the birth and AGD measures of their child at enrollment, making selection bias less possible. We adjusted for relevant confounders but cannot rule out the possibility of confounding by other factors associated with pesticide exposure and growth measures, e.g. co-exposure to other environmental chemicals or lifestyle factors associated with pesticide exposure including potential beneficial effects of intake of fruit and vegetables which might have introduced negative confounding [56]. An additional limitation is that the pesticide metabolite concentrations were determined by one single spot urine sample collected after overnight fasting. The pesticides are assumed to be rapidly metabolized and excreted from the body within hours to days and substantial within-subject variability has been demonstrated for organophosphate metabolites [57] and to a lesser degree for pyrethroids [58]. Thus, some exposure misclassification is expected but it is unlikely to be dependent on the investigated health outcomes, making it non-differential and tend to bias the effect estimates toward the null. By dividing the continuous exposure variables into categorical variables (tertiles), we tried to reduce potential misclassification by assuming that individuals with a general high intake of non-organic fruit and vegetables are more likely to be in the upper tertile compared to those with lower intake. This assumption is supported by a previous study, showing that a single urine measure of TCPY was valid to predict tertiles of exposure [59]. Finally, we performed a relatively large number of statistical analyses, which increase the likelihood of chance findings.

5. Conclusion

In this population-based study of 858 mother-child pairs we found no consistent dose–response associations between maternal urinary concentrations of pesticide metabolites and birth outcomes or AGD in the offspring. However, the tendency towards a dose-related elonga-

tion of AGD in girls related to 3-PBA and DAP deserves to be further investigated since the exposure to pyrethroids and OPs is widespread and even weak effects might add to the combined effect of environmental endocrine disruptors on reproductive development.

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Table 1. Concentrations of 2,4-D, 3-PBA, TCPY, and ∑DAP, ∑DE, ∑DM* measured in urine samples collected in gestational week 28 from pregnant women in the Odense Child Cohort (2010-2012)

Pesticide metabolite	N	LOD	%>LOD	Minimum	5	25	50	75	95	Maximum
2,4 D (µg/L)	858	0.03	97.6	<LOD	0.04	0.10	0.16	0.28	0.70	10.16
3-PBA (µg/L)	858	0.03	94.3	<LOD	<LOD	0.93	0.20	0.49	2.17	75.96
TCPY (µg/L)	858	0.3	93.1	<LOD	<LOD	0.95	1.73	3.11	8.15	65.91
∑DAP (nmol/L)	564			12.23	12.23	26.89	56.5	102.89	252.81	1446.16
∑DE (nmol/L)	564			3.76	3.76	10.88	20.05	45.02	108.52	334.22
∑DM (nmol/L)	564			8.47	8.47	8.47	25.64	54.35	168.06	1416.83
2,4 D (µg/g creatinine)	857			0.007	0.05	0.11	0.18	0.30	0.73	5.52
3-PBA (µg/g creatinine)	857			0.007	0.04	0.13	0.23	0.45	1.73	29.34
TCPY (µg/g creatinine)	857			0.07	0.51	1.15	1.84	3.15	8.26	74.16
∑DAP (nmol/g creatinine)	564			7.16	16.61	36.55	58.42	103.7	259.06	1144.52
∑DE (nmol/g creatinine)	564			1.53	4.18	13.12	23.26	41.85	95.35	346.94
∑DM (nmol/g creatinine)	564			2.96	5.84	14.23	29.70	57.23	173.23	1121.31

LOD, Limit of Detection

*∑DE= DEP + DEDTP + DEDTP , ∑DM = DMP + DMTP + DMDTP , ∑DAP=∑DE+ ∑DM

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Pesticide exposure and health risk in susceptible population groups

Summary

We previously reported associations between pesticide exposure during early pregnancy in female greenhouse workers and their children's development and growth, including more body fat and higher blood pressure at school age. This association was mainly seen for children with a common gene variant (change in genetic material), which nearly half of the children had. One aim of this project was to investigate a possible mechanism behind this finding. We found that children who had the gene variant and were exposed to pesticides in foetal life, had a different methylation pattern in genes involved in regulation of appetite and energy balance than unexposed children or children without the gene variant. This indicates that the activity of these genes has been affected by pesticide exposure in early foetal life.

Another aim was to investigate the pesticide exposure level among pregnant women and schoolchildren. We measured degradation products of insecticides (organophosphates and pyrethroids) and the herbicidal 2,4-D in urine samples and found measurable amounts in more than 90% of the samples. The level was generally higher or at the same level as in studies from other countries. We saw no clear association between the women's urine concentrations of the pesticides and their children's birth weight and length or their head and abdominal circumference. There was a tendency for a longer ano-genital distance at 3 months in the girls related to the insecticides and shorter distance in the boys related to 2,4-D. This may indicate a weak disturbance of the children's sexual development. Finally, we investigated whether the urine concentration of insecticides in school age children was related to motor function and attention. We found no correlation for pyrethroids, but organophosphates were related to a reduced attention in girls.

Resume

Vi har tidligere fundet sammenhæng mellem gartneriansatte kvinders udsættelse for pesticider tidligt i graviditeten og deres børns udvikling og vækst, herunder mere kropsfedt og højere blodtryk ved skolealderen. Denne sammenhæng sås primært for børn med en hyppig genvariant (ændring i arvematerialet), som næsten halvdelen af børnene havde. Et formål med dette projekt var at undersøge en mulig mekanisme bag dette fund. Vi fandt, at børn med den pågældende genvariant, som havde været udsat for pesticider som fostre, havde et andet metyleringsmønster i gener involveret i regulering af appetit og energiomsætning end ueksponerede børn eller børn uden den pågældende genændring. Det tyder på at disse generes aktivitet er ændret af den tidlige pesticideksponering.

Et andet formål var at undersøge eksponeringsniveauet for pesticider blandt gravide kvinder og skolebørn. Vi målte nedbrydningsprodukter af insektmidler (organofosfater og pyrethroider) og ukrudtsmidlet 2,4-D i urinprøver og fandt målbare mængder i mere end 90 % af prøverne. Niveauet var generelt højere eller på samme niveau som i undersøgelser fra andre lande. Vi fandt ingen tydelige sammenhænge mellem kvindernes urinkoncentrationer af pesticiderne og deres børns fødselsvægt og -længde eller hoved- og maveomfang. Der var en tendens til længere ano-genital afstand ved 3 måneder hos pigerne relateret til insektmidlerne og kortere afstand hos drengene relateret til 2,4-D. Det kan indikere en svag forstyrrelse af børnenes kønsudvikling. Endelig undersøgte vi, om urinkoncentrationen af insektmidler hos skolebørn var relateret til motoriske funktion og koncentrationsevne. Vi fandt ingen sammenhæng for pyrethroider men organofosfater var relateret til en nedsat koncentrationsevne hos pigerne.

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