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Prenatal Pesticide Exposure Associated with Glycated Haemoglobin and Markers of Metabolic Dysfunction in Adolescents

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Abstract

Background: Pesticide exposure has been associated with increased risk of diabetes mellitus in adults, but potential effects of prenatal exposure on glucose regulation have not been investigated. The aim of this study was to investigate if maternal occupational pesticide exposure in pregnancy was associated with glycated haemoglobin A1c (HbA1c) in adolescents and whether an association was modified by sex and paraoxonase-1 (*PON1*) Q192R polymorphism.

Methods: A prospective cohort study of children whose mothers were either occupationally exposed or unexposed to pesticides in early pregnancy. At age 10-to-16 years, the children (n=168) underwent clinical examinations including pubertal stage assessment (accepted by 141 children) and blood sampling. *PON1* Q192R genotype was available for 139 children and 103 mothers. The main outcome measure was HbA1c but other relevant biomarkers were also included.

Results: Prenatal pesticide exposure was associated with a 5.0% (95% confidence interval: 1.8; 8.2) higher HbA1c compared to unexposed children after adjustment for confounders. After stratification, the association remained significant for girls (6.2% (1.6; 11.1)) and if the child or the mother had the *PON1* 192R-allele (6.1% (1.6; 10.8) and 7.1% (2.0; 12.6), respectively). Besides, an exposure-related increase was seen for the leptin-to-adiponectin ratio, for plasminogen activator inhibitor type-1 in girls, and for interleukin-6 in children whose mothers had the R-allele.

Conclusion: Prenatal pesticide exposure was associated with higher HbA1c and changes in related biomarkers in adolescents. Our results suggest an adverse effect on glucose homeostasis and support previous findings from this cohort of an exposure-associated metabolic risk profile with higher susceptibility related to female sex and the *PON1* 192R-allele.

Keywords: HbA1c, pesticides, paraoxonase-1, prenatal exposure, metabolic biomarkers

Funding: The study was supported by The Danish Environmental Protection Agency as part of the Danish Centre on Endocrine Disruptors (grant number MST-621-00065).

Ethics: The study was conducted according to the Helsinki II Declaration with written informed consent by one or both parents as approved by The Regional Scientific Ethical Committees for Southern Denmark (S-20070068) and The Danish Data Protection Agency (1996-1200-154, 2007-41-0956).

Declarations of interest: none

1. Introduction

Exposure to pesticides has been associated with an increased risk of diabetes mellitus (Evangelou et al., 2016). The evidence was strongest for persistent organochlorine pesticides, but occupational exposure to currently used non-persistent pesticides has also been associated with abnormal glucose regulation and increased risk of type 2 diabetes (T2D) in adults (Schreinemachers, 2010; Xiao et al., 2017). In experimental studies, early life exposure to low doses of some commonly used pesticides caused disrupted glucose and lipid homeostasis in adult rats (Hocine et al., 2016; Lassiter et al., 2010; Slotkin, 2011) but the potential impact of prenatal exposure on glucose homeostasis later in life has not been explored in human studies.

To investigate the potential health effects of prenatal pesticide exposure, we have followed a cohort of children whose mothers were employed in greenhouse horticulture during 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. In this cohort we found an association between maternal pesticide exposure and body fat accumulation from birth to school age (6-11 years of age) in their children (Wohlfahrt-Veje et al., 2011). This association was mainly driven by children with a single nucleotide polymorphism in the paraoxonase-1 gene (*PON1*).

Paraoxonase-1 is a high-density lipoprotein (HDL)-associated enzyme with antioxidant functions. A common polymorphism in the coding sequence, a glutamine (Q)/arginine (R) substitution at position 192, affects the antioxidant properties (Mackness and Mackness, 2015) and the R-allele has been associated with higher risk for coronary heart disease (Wang et al., 2011). We found that prenatally pesticide exposed children with the R-allele had larger abdominal circumference and higher body fat content, blood pressure, and serum concentrations of selected metabolic markers than unexposed children with the same genotype (Andersen et al., 2012; Jorgensen et al., 2015).

The findings on body fat composition were confirmed by dual X-ray absorptiometry (DXA) at adolescence where especially android fat%, but also gynoid and total fat%, were positively associated with prenatal pesticide exposure. These associations were stronger for girls than for boys and also stronger if the child or the mother carried the R-allele (Tinggaard et al., 2016). Thus, our findings indicate disturbance of metabolic pathways in the children, and we therefor hypothesized that glucose regulation would also be impaired, possibly dependent on sex and *PON1* Q192R genotype. Glycated hemoglobin A1c (HbA1c) reflects average blood glucose concentration over the previous eight to 12 weeks (Nathan et al., 2007). To test the hypothesis, we investigated associations between prenatal pesticide exposure and HbA1c in blood samples collected at two clinical examinations at ages 10-15 and 11-16 years. To support our findings, we also measured metabolic and inflammatory biomarkers related to insulin sensitivity.

2. Methods

2.1. Study population

From 1996 to 2000 pregnant women working in greenhouse nurseries were recruited consecutively when they were referred to the Department of Occupational Health at Odense University Hospital, Denmark, for risk assessment of their working conditions and guidance for safe work practices during pregnancy. Their children (N=203) were first examined at three months of age (Andersen et al., 2008) and then followed up at school age where 44 additional age-matched controls were included (Wohlfahrt-Veje et al., 2011). The present study includes data from two additional follow-up examinations during puberty when the children were between 10 and 16 years of age (Tinggaard et al., 2016).

Details of the study, including recruitment procedure and exposure assessment, have been described previously (Andersen et al., 2008; Wohlfahrt-Veje et al., 2011). Briefly, the mothers were categorized as occupationally exposed or unexposed to pesticides based on detailed information about working conditions for the previous three months obtained from interview at enrollment (gestational weeks 4-10) and supplemented by telephone interview of the employers. All exposure assessments were performed independently by two toxicologists with expertise in working conditions in greenhouse horticultures and completed before the first examination of the children. Women categorized as 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. For all women, the main work functions were nursing and handling of plants, which had some times been treated with pesticides. More than 100 different pesticide formulations were used in the greenhouses and the women were often exposed to mixtures of various insecticides, fungicides, and growth regulators.

Information on life-style factors during pregnancy was collected by an interview-assisted questionnaire at the first examination of the child. Socioeconomic status (SES) was grouped into five groups ranked 1(high) to 5 (low) based on parental education and occupation (Hansen, 1978). The group of the highest ranked parent living with the child was used. Information on gestational age at birth, birth weight, and birth length was obtained from obstetric records. Weight-for-gestational age (WGA) was calculated according to Marsal (Marsal et al., 1996).

Invitations for this study were sent to 243 eligible children/families (one child had died and three families had moved abroad). Overall, 168 children participated in at least one of two follow-up examinations that took place from October 2011 to January 2012 and from March to June 2013.

The study was conducted according to the Helsinki II Declaration with written informed consent by one or both parents as approved by The Regional Scientific Ethical Committees for Southern Denmark (S-20070068) and The Danish Data Protection Agency (1996-1200-154, 2007-41-0956).

2.2. Clinical examination and blood sampling

The clinical examination included anthropometry and assessment of pubertal stage according to Tanner and Marshall as previously described (Tinggaard et al., 2016). Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Total body fat percentage (body fat%) was calculated from skinfolds by the Slaughter equation as previously described (Tinggaard et al., 2016). The same paediatrician (JT) performed all clinical examinations blinded to information about maternal pesticide exposure.

Venous blood samples were collected in EDTA-coated and uncoated vials. Erythrocyte fractions separated from EDTA-treated samples and serum separated from uncoated vials were stored at $-80^{\circ}C$ until analysis. Blood samples were obtained from 157 children and from 122 of their mothers. In an attempt to get overnight fasting samples, we tried to schedule one of the two examinations to take place in the morning, either before school start or in weekends. This way, we obtained fasting blood samples from 124 children.

One child with diagnosed type 1 diabetes mellitus was excluded from the study. Since puberty affects insulin resistance and related metabolic biomarkers (Hannon et al., 2006), 15 children who

did not accept assessment of pubertal stage were also excluded, thus leaving 141 children with available blood samples.

2.3 Laboratory analyses

2.3.1 *PON1* genotyping

DNA was isolated from buffy coats and *PON1* Q192R (rs662) was genotyped by a Taqman-based allelic discrimination assay as previously described (Christiansen et al., 2004). The *PON1* genotype was successfully determined in 122 mothers and 40 additional children for whom the genotype was not determined at previous examinations.

2.3.2 Measurement of HbA1c and metabolic and inflammatory biomarkers

Besides HbA1c, we selected biomarkers identified a priori to be associated with insulin sensitivity (Festa et al., 2002; Finucane et al., 2009; Friedrich et al., 2012) including markers of glucose regulation (insulin, c-peptide, insulin-like growth factor 1 (IGF-1)), adipocyte function (leptin, adiponectin), inflammation (interleukin 6 (IL6), tumour necrosis factor alpha (TNF α), high-sensitivity C-reactive protein (hs-CRP), plasminogen activator inhibitor type-1 (PAI-1)), and dyslipidaemia (total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides (TG)).

HbA1c was analysed in the erythrocyte fraction using high performance liquid chromatography (HPLC) (TOSOH G8) with inter-assay CV below 5%. All other biomarkers were analysed in serum. Insulin, c-peptide, leptin, adiponectin, TNF α , and IL6 were determined by quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kits (Quantikine, R&D Systems, Minn., USA) and PAI-1 by an ELISA kit (Invitrogen from Thermo Fisher, Carlsbad, Ca, USA). All inter-

assay CVs were below 10%. IGF-1 was analysed by chemiluminescence immunoassay (IDS-iSYS) with inter-assay CV below 7.2%. Hs-CRP was analysed by immunoturbidimetry using a Roche/Hitachi Modular P Analyzer ACN 217(Tina-Quant, Roche/Hitachi, Mannheim, Germany), with an inter-assay CV below 6%. Total-cholesterol, HDL, LDL, and TG were measured by enzymatic colorimetric methods using a Roche/Hitachi Modular PD Analyzer ACN 433 (Roche/Hitachi, Mannheim, Germany) with inter-assay CVs below 3%.

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

2.4 Data Analyses

Since only 9 children and 6 mothers were *PON1* 192RR homozygous, carriers of the R-allele were combined (QR and RR genotypes) in the data analyses.

For biomarkers that were potentially affected by fasting (insulin, c-peptide, PAI-1, HDL, LDL, cholesterol, and TG), only measurements from samples obtained after fasting were included in the data analyses. The leptin/adiponectin ratio (LAR) was calculated since LAR was reported to be a better indicator of insulin resistance than the single adipokines (Finucane et al., 2009).

Age and sex specific standard deviation scores (SDS) for BMI, height, and weight were calculated using a Danish reference population (Tinggaard et al., 2016). Differences in characteristics between

exposed and unexposed children were tested by one-way analysis of variance (continuous variables) and Fisher's exact test (binary variables) or Likelihood Ratio (variables with more than two categories). Differences in concentrations of biomarkers between exposed and unexposed children and between girls and boys were tested by Mann-Whitney U-test.

We first performed sex and puberty adjusted partial correlation analyses to establish the associations between HbA1c and BMI SDS, body fat%, WGA, and the other biomarkers. Differences in biomarker concentrations (except for hs-CRP) between exposed and unexposed children were estimated by standard multiple regression analysis. To obtain variance homogeneity and normal distribution of residuals, body fat% and the biomarker concentrations were transformed using the natural logarithm. However, for hs-CRP a normal distribution was not achievable and therefore hs-CRP was converted to a binary variable (0: hs-CRP \leq 0.20 mg/L (median); 1: hs-CRP $>$ 0.20 mg/L). Putative confounders were identified from a priori considerations and included sex and age at examination, SES, and pubertal development (yes/no). SES was re-coded into three categories: groups 1-3, group 4 or group 5 with group 4 as reference as most families belonged to this group. Maternal smoking in pregnancy was considered but not included because of correlation with SES and similar distribution between unexposed and exposed mothers. Estimates of associations were converted to express the relative difference in percent with 95% confidence intervals (95% CI) between exposed and unexposed children for the outcome variables. The odds ratio for hs-CRP above the median for exposed children was calculated using logistic regression and the same set of covariates as for the other outcomes. A significance level of 5% was used in all analysis.

We investigated potential effect modification by child sex and by child and maternal *PON1* Q192R genotype using stratification and interaction cross-product terms. Because birth weight and body fat content may be mediators of associations between prenatal pesticide exposure and the outcome variables, regression analyses were repeated with WGA or body fat% in the models. Body fat% calculated from skinfolds was used in the data analysis because DXA was performed only at the first of the two follow-up examinations.

We performed several sensitivity analyses. We repeated the analyses after excluding children with BMI Z-scores below -2 or above +2. Since puberty markedly affected most biomarkers, analyses were repeated using Tanner Stages 1 to 5 instead of the binary variable as well as after excluding 11 pre-pubertal (Tanner stage 1) children. Considerably more of the unexposed children recruited at the first follow-up (at 6 to 11 years-of-age) belonged to social class 1-3 compared to children from the original birth cohort (54.5% vs 18.8%) and therefore we repeated the regression analyses with only unexposed control children from the original birth cohort (18 out of 53 unexposed children).

3. Results

This study included 141 adolescents who accepted pubertal stage assessment and for whom a blood sample was obtained. Seventy-four blood samples (46 fasting, 28 non-fasting) were obtained at the first examination and 67 samples (65 fasting, 2 non-fasting) at the second examination. The mean age of the children at the time of blood sampling was 13.0 years (range: 9.9–16.2 years). Children prenatally exposed to pesticides had greater body weight, body fat%, BMI, and BMI SDS than unexposed children (Table 1). Although not statistically significant, exposed children also had larger waist circumference, lower WGA, and higher blood pressure. They tended to be older, from

families with lower SES, and a higher fraction of exposed boys had entered puberty as compared to the unexposed boys.

The median HbA1c was 32.0 mmol/mol and was slightly lower in girls (32.0 mmol/mol) than in boys (33.0 mmol/mol). Children who had not entered puberty had lower median HbA1c than pubertal children (31.0 vs 33.0 mmol/mol). All HbA1c values (min-max: 24-39 mmol/mol) were within the reference range for non-diabetic adults (Weykamp, 2013).

After controlling for sex and puberty, HbA1c was weakly correlated to IL-6 ($r=0.21$, $p=0.02$) but not to any other of the biomarkers, BMI SDS, body fat%, or WGA.

In crude analyses, HbA1c was significantly higher among prenatally exposed children than unexposed children (Table 2) and reflected a shift of the distribution curve towards higher levels among the exposed children (Figure 1). The difference remained significant after adjustment for sex, age, puberty, and SES. The relative mean difference (β) in HbA1c between exposed and unexposed children was 5.0% (95% CI: 1.8%; 8.2%, $p=0.002$) corresponding to a difference in adjusted geometric mean of 1.6 mmol/mol. After stratification, the association was significant in girls ($\beta=6.2\%$, $p=0.008$) but not in boys ($\beta=3.9\%$, $p=0.07$) (Table 3) and significant if the child or mother had the *PON1* 192R-allele ($\beta=6.1\%$ and 7.1% , respectively, $p\leq 0.007$ for both) (Table 4).

Exposure-related differences were also seen for leptin, adiponectin, and LAR (Table 2). After adjustment for confounders, LAR remained significantly higher among exposed children but the difference did not reach statistical significance in sex-stratified analyses (Table 3). In exposed girls,

PAI-1 was significantly higher and a tendency towards lower insulin and higher TG was seen compared to unexposed girls. Exposed boys tended to have lower TNF- α than unexposed boys but the difference was reduced after excluding three pre-pubertal boys (β =-4.7% (95% CI: -14.6%; 6.3%)). Heterogeneity related to maternal or child PON1 Q192R genotype was indicated for several biomarkers other than HbA1c (Table 4). Exposed children with the R-allele, or children whose mothers had the R-allele, had significantly higher LAR and leptin concentrations than unexposed children within the same strata. An exposure-related increase in IL-6 was seen for children whose mothers were R-allele carriers. A similar tendency was seen for children with the R-allele. For the lipid biomarkers, exposed children with the R-allele tended to have higher TG and lower HDL concentrations than unexposed children. Exposed children with the QQ genotype had significantly lower serum concentrations of IGF-1 and insulin than unexposed children. Dependence of the maternal genotype was less clear, but IGF-1 tended to be higher in exposed children of QQ homozygous mothers but lower in exposed children of mothers with the R-allele compared to unexposed children.

The association between prenatal pesticide exposure and HbA1c remained significant in sensitivity analyses (Table 5) and after including body fat% in the models. Associations between exposure and LAR were attenuated after including body fat% (for all children: β =10.4% (95% CI: -19.5%; 51.5%)). No other associations were markedly affected by the adjustment. Finally, using Tanner Stages 1 to 5 instead of the binary variable (puberty (yes/no)) or including WGA in the models changed the results to a negligible extent only.

4. Discussion

In this longitudinal study, maternal occupational pesticide exposure in early pregnancy was associated with higher HbA1c in the offspring at adolescence thus indicating a long-term effect on glucose homeostasis. The association remained significant after adjusting for body fat%. In addition, exposed children had significantly higher LAR and they tended to have higher IL-6 and PAI-1 all of which are related to insulin resistance, increased risk of T2D, and metabolic syndrome (Festa et al., 2002; Finucane et al., 2009; Yeste et al., 2007). After stratification for sex or *PON1* genotype, some of these associations seemed strengthened among girls and if the child or mother had the *PON1* 192R-allele although the p-values for interaction were above 0.05. An exposure-related tendency toward higher TG and lower HDL cholesterol for girls and R-carriers also points to an alteration in lipid profile consistent with development of insulin resistance (Yeste et al., 2007). Thus, occupational exposure to pesticides early in pregnancy may have long-lasting effects on metabolic regulation and risk profile, especially among girls and if the child or the mother carries the *PON1* 192R-allele.

The exposure-related differences in HbA1c seen in this study were modest, and all HbA1c values were well below the cut-point of 48 mmol/mol (6.5%) recommended by the WHO for diagnosing diabetes mellitus in adults and likewise the 42 mmol/mol as cut-point for high diabetes risk (WHO, 2011). However, the applicability of these cut-points among adolescents is unclear. Some studies indicate that lower HbA1c values are required for children and adolescents to prevent increased risk of subsequent diabetes mellitus development and complications related to hyperglycemia (Chan et al., 2015).

Although pesticide exposure has been associated with increased risk of T2D in adults and abnormal glucose regulation in both epidemiological and experimental studies (Evangelou et al., 2016; Xiao et al., 2017) no previous studies have, to our knowledge, investigated associations between exposures to currently used pesticides during pregnancy and glucose homeostasis in the offspring later in life. As supportive evidence, a recent birth cohort study from the general French population found a positive association between maternal urinary concentrations of organophosphate metabolites in early pregnancy and insulin concentrations in cord blood serum (Debost-Legrand et al., 2016). Our findings are also supported by experimental studies. In rats, perinatal low-dose exposure to organophosphate insecticides caused disrupted glucose and lipid homeostasis, and excess weight gain in adulthood (Lassiter et al., 2010; Slotkin, 2011). Interestingly, some of these effects occurred in a sex-specific manner with greater sensitivity among females (Lassiter et al., 2008). Similar effects were reported after low-dose gestational exposure to the pyrethroid insecticide alpha-cypermethrin (Hocine et al., 2016). A recent study, investigated long-term effects of gestational and lactational exposure to a low dose mixture of six pesticides that previously was found to cause lower birth weight. No effects on glucose tolerance and insulin resistance were observed but in male offspring the weight of fatty tissue was higher relative to body weight and female offspring displayed elevated leptin levels (Svingen et al., 2018).

The mechanisms whereby some pesticides might promote T2D development are largely unknown but suggested mechanisms from experimental studies include disturbance of mitochondrial functions and cell signaling cascades (Slotkin, 2011; Xiao et al., 2017). Many pesticides have endocrine disrupting and/or neurotoxic properties (Andersen et al., 2002; Bjorling-Poulsen et al., 2008; Zhang et al., 2016) and exposure during critical developmental windows may permanently disrupt neuroendocrine signaling and functions (Parent et al., 2011; Waye and Trudeau, 2011).

Some pesticides have been demonstrated *in vitro* to interfere with actions of glucocorticoids (Slotkin et al., 2012; Zhang et al., 2016) and, hence, might affect hypothalamic-pituitary-adrenal (HPA) axis development. Recently, the fungicide tolylfluanid was reported to increase adipocyte glucocorticoid receptor signaling and to promote weight gain, higher total fat mass, higher LAR, glucose intolerance, and increased insulin resistance in mice after few weeks low-dose exposure (Regnier et al., 2015). Besides, pesticides that can interfere with sex-hormone function (Andersen et al., 2002) may disturb the sex-dimorphic expression of oestrogen receptors and oestrogen-responsive genes in the hypothalamus and thereby affect metabolic regulation in a sex-specific manner (Rebuli and Patisaul, 2016). Thus, these mechanisms might explain the stronger association between exposure and metabolic disturbances in girls observed both in our study and in several other studies of early exposures to endocrine disrupting chemicals (Russ and Howard, 2016).

In line with our previous findings in this cohort, the association between exposure and HbA1c seemed to be dependent on maternal and child *PON1* Q192R genotype (Andersen et al., 2012; Tinggaard et al., 2016). We recently found a differential methylation profile in several genes involved in neuroendocrine signaling pathways controlling appetite and energy balance in DNA isolated from blood samples from exposed children with the R-allele compared to exposed children with the QQ-genotype or unexposed children (Declerck et al., 2017). Thus, the higher susceptibility related to the R-allele might be mediated by epigenetic metabolic programming. Besides, the *PON1* Q192R polymorphism determines the anti-oxidative and anti-inflammatory properties of the enzyme with lower efficiency linked to the R-allele (Mackness and Mackness, 2015). In mice, over-expression of human *PON1* was reported to prevent diabetes development apparently through its antioxidant properties and by stimulating β -cell insulin release (Koren-Gluzer et al., 2011). 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 (Alegria-Torres et al., 2015). Since gestational diabetes is known to be associated with higher HbA1c and is a risk factor for diabetes development in the offspring (Jansen et al., 2015), it is conceivable that glucose regulation in the mothers, especially those with an R-allele, may have been disturbed by their occupational pesticide exposure and consequently affected the metabolic regulation in their children. We do not, however, have HbA1c measurements from the mothers to explore this possibility.

Besides its antioxidant property, PON1 also hydrolyses a range of substrates, including organophosphate insecticides. However, at relatively low exposure levels, as in this study, the capacity to detoxify organophosphates is considered to be independent of the *PON1* Q192R genotype (Coombes et al., 2014).

Our study has some limitations. To investigate glucose regulation in pubertal children is challenging, since puberty per se is associated with a modest degree of insulin resistance and related changes in metabolic biomarkers (Hannon et al., 2006). Since more exposed than unexposed children had entered puberty, this difference may have affected the results. The exposure-associated difference in HbA1c was, however, not diminished after adjusting for Tanner stages or after exclusion of 11 pre-pubertal (Tanner stage 1) children. Another limitation of the study is that we cannot identify individual pesticides related to the findings. Overall, the working conditions were well controlled and the women were seldom involved in mixing or applying insecticides. The main work function was nursing and handling of plants, which often had been treated with pesticides and the women were in general exposed to mixtures of various pesticides (Andersen et al., 2008). The small sample size, especially in analyses stratified by sex or *PON1* Q192R genotype, is also a limitation and impairs our ability to detect interactions between exposure and sex or *PON1*

polymorphism, respectively. However, the consistent finding of more pronounced effects related to the R-allele and to females (Andersen et al., 2012; Tinggaard et al., 2016) suggest a true association. Out of 243 eligible children, 141 (58%) were included in the study. A lower fraction of the included children had mothers who smoked (21.7% vs 37.5%) or were occupationally exposed to pesticides in pregnancy (62.4% vs 75.5%) and they tended to have higher birth weight (mean 3602 g vs 3457 g) compared to children who were not included. Thus, we cannot exclude potential selection bias introduced by loss of children to follow-up. Also inclusion of additional controls at the first follow-up at age 6-11 might have introduced selection bias. However, the exposure-related difference in HbA1c was apparent even after excluding these additional controls.

In conclusion, our results suggest an adverse effect of prenatal pesticide exposure on glucose homeostasis and markers related to insulin resistance (LAR, PAI-1, IL-6) in adolescents. The results support previous findings from this cohort of an exposure-associated adverse metabolic risk profile, along with a higher susceptibility in girls and a genetic predisposition linked to the *PON1* 192R-allele. These findings relate to occupational pesticide exposure but the working conditions were well controlled, none of the mothers were considered highly exposed, and the exposure stopped or was minimized after confirmation of pregnancy in the first trimester. Thus, the relevance of these results in relation to pesticide exposure levels occurring in the general population deserves to be explored, since reduction of factors contributing to the epidemic of obesity and related metabolic diseases would be of great public health importance.

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References

- Alegria-Torres, J. A., Garcia-Dominguez, M. L., Cruz, M., Aradillas-Garcia, C., 2015. Q192R polymorphism of paraoxonase 1 gene associated with insulin resistance in Mexican children. *Arch Med Res.* 46, 78-83.
- Andersen, H. R., Schmidt, I. M., Grandjean, P., Jensen, T. K., Budtz-Jorgensen, E., Kjaerstad, M. B., Baelum, J., Nielsen, J. B., Skakkebaek, N. E., Main, K. M., 2008. Impaired reproductive development in sons of women occupationally exposed to pesticides during pregnancy. *Environ Health Perspect.* 116, 566-72.
- Andersen, H. R., Vinggaard, A. M., Rasmussen, T. H., Gjermansen, I. M., Bonefeld-Jorgensen, E. C., 2002. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. *Toxicol Appl Pharmacol.* 179, 1-12.
- Andersen, H. R., Wohlfahrt-Veje, C., Dalgard, C., Christiansen, L., Main, K. M., Nellemann, C., Murata, K., Jensen, T. K., Skakkebaek, N. E., Grandjean, P., 2012. Paraoxonase 1 polymorphism and prenatal pesticide exposure associated with adverse cardiovascular risk profiles at school age. *PLoS One.* 7, e36830.
- Bjorling-Poulsen, M., Andersen, H. R., Grandjean, P., 2008. Potential developmental neurotoxicity of pesticides used in Europe. *Environ Health.* 7, 50.
- Chan, C. L., Pyle, L., Newnes, L., Nadeau, K. J., Zeitler, P. S., Kelsey, M. M., 2015. Continuous glucose monitoring and its relationship to hemoglobin A1c and oral glucose tolerance testing in obese and prediabetic youth. *J Clin Endocrinol Metab.* 100, 902-10.
- Christiansen, L., Bathum, L., Frederiksen, H., Christensen, K., 2004. Paraoxonase 1 polymorphisms and survival. *Eur J Hum Genet.* 12, 843-7.
- Coombes, R. H., Meek, E. C., Dail, M. B., Chambers, H. W., Chambers, J. E., 2014. Human paraoxonase 1 hydrolysis of nanomolar chlorpyrifos-oxon concentrations is unaffected by phenotype or Q192R genotype. *Toxicol Lett.* 230, 57-61.
- Debost-Legrand, A., Warembourg, C., Massart, C., Chevrier, C., Bonvallot, N., Monfort, C., Rouget, F., Bonnet, F., Cordier, S., 2016. Prenatal exposure to persistent organic pollutants and organophosphate pesticides, and markers of glucose metabolism at birth. *Environ Res.* 146, 207-17.
- Declerck, K., Remy, S., Wohlfahrt-Veje, C., Main, K. M., Van Camp, G., Schoeters, G., Vanden Berghe, W., Andersen, H. R., 2017. 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. *Clin Epigenetics.* 9, 35.
- Evangelou, E., Ntritsos, G., Chondrogiorgi, M., Kavvoura, F. K., Hernandez, A. F., Ntzani, E. E., Tzoulaki, I., 2016. Exposure to pesticides and diabetes: A systematic review and meta-analysis. *Environ Int.* 91, 60-68.
- Festa, A., D'Agostino, R., Jr., Tracy, R. P., Haffner, S. M., 2002. Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes: the insulin resistance atherosclerosis study. *Diabetes.* 51, 1131-7.
- Finucane, F. M., Luan, J., Wareham, N. J., Sharp, S. J., O'Rahilly, S., Balkau, B., Flyvbjerg, A., Walker, M., Hojlund, K., Nolan, J. J., Savage, D. B., 2009. Correlation of the leptin:adiponectin ratio with measures of insulin resistance in non-diabetic individuals. *Diabetologia.* 52, 2345-9.
- Friedrich, N., Thuesen, B., Jorgensen, T., Juul, A., Spielhagen, C., Wallaschofski, H., Linneberg, A., 2012. The association between IGF-I and insulin resistance: a general population study in Danish adults. *Diabetes Care.* 35, 768-73.
- Hannon, T. S., Janosky, J., Arslanian, S. A., 2006. Longitudinal study of physiologic insulin resistance and metabolic changes of puberty. *Pediatr Res.* 60, 759-63.
- Hansen, E. J., 1978. Distribution of living conditions. Danish National Institute of Social Research, Copenhagen.

- Hocine, L., Merzouk, H., Merzouk, S. A., Ghorzi, H., Youbi, M., Narce, M., 2016. The effects of alpha-cypermethrin exposure on biochemical and redox parameters in pregnant rats and their newborns. *Pestic Biochem Physiol.* 134, 49-54.
- Jansen, H., Wijga, A. H., Scholtens, S., Koppelman, G. H., Postma, D. S., Brunekreef, B., de Jongste, J. C., Smit, H. A., Stolk, R. P., 2015. Change in HbA1c levels between the age of 8 years and the age of 12 years in Dutch children without diabetes: the PIAMA birth cohort study. *PLoS One.* 10, e0119615.
- Jorgensen, A., Nellemann, C., Wohlfahrt-Veje, C., Jensen, T. K., Main, K. M., Andersen, H. R., 2015. Interaction between paraoxonase 1 polymorphism and prenatal pesticide exposure on metabolic markers in children using a multiplex approach. *Reprod Toxicol.* 51, 22-30.
- Koren-Gluzer, M., Aviram, M., Meilin, E., Hayek, T., 2011. The antioxidant HDL-associated paraoxonase-1 (PON1) attenuates diabetes development and stimulates beta-cell insulin release. *Atherosclerosis.* 219, 510-8.
- Lassiter, T. L., Ryde, I. T., Levin, E. D., Seidler, F. J., Slotkin, T. A., 2010. Neonatal exposure to parathion alters lipid metabolism in adulthood: Interactions with dietary fat intake and implications for neurodevelopmental deficits. *Brain Res Bull.* 81, 85-91.
- Lassiter, T. L., Ryde, I. T., Mackillop, E. A., Brown, K. K., Levin, E. D., Seidler, F. J., Slotkin, T. A., 2008. Exposure of neonatal rats to parathion elicits sex-selective reprogramming of metabolism and alters the response to a high-fat diet in adulthood. *Environ Health Perspect.* 116, 1456-62.
- Mackness, M., Mackness, B., 2015. Human paraoxonase-1 (PON1): Gene structure and expression, promiscuous activities and multiple physiological roles. *Gene.* 567, 12-21.
- Marsal, K., Persson, P. H., Larsen, T., Lilja, H., Selbing, A., Sultan, B., 1996. Intrauterine growth curves based on ultrasonically estimated foetal weights. *Acta Paediatr.* 85, 843-8.
- Nathan, D. M., Turgeon, H., Regan, S., 2007. Relationship between glycated haemoglobin levels and mean glucose levels over time. *Diabetologia.* 50, 2239-44.
- Parent, A. S., Naveau, E., Gerard, A., Bourguignon, J. P., Westbrook, G. L., 2011. Early developmental actions of endocrine disruptors on the hypothalamus, hippocampus, and cerebral cortex. *J Toxicol Environ Health B Crit Rev.* 14, 328-45.
- Rebuli, M. E., Patisaul, H. B., 2016. Assessment of sex specific endocrine disrupting effects in the prenatal and pre-pubertal rodent brain. *J Steroid Biochem Mol Biol.* 160, 148-59.
- Regnier, S. M., Kirkley, A. G., Ye, H., El-Hashani, E., Zhang, X., Neel, B. A., Kamau, W., Thomas, C. C., Williams, A. K., Hayes, E. T., Massad, N. L., Johnson, D. N., Huang, L., Zhang, C., Sargis, R. M., 2015. Dietary exposure to the endocrine disruptor tolylfluanid promotes global metabolic dysfunction in male mice. *Endocrinology.* 156, 896-910.
- Russ, K., Howard, S., 2016. Developmental Exposure to Environmental Chemicals and Metabolic Changes in Children. *Curr Probl Pediatr Adolesc Health Care.* 46, 255-85.
- Schreinemachers, D. M., 2010. Perturbation of lipids and glucose metabolism associated with previous 2,4-D exposure: a cross-sectional study of NHANES III data, 1988-1994. *Environ Health.* 9, 11.
- Slotkin, T. A., 2011. Does early-life exposure to organophosphate insecticides lead to prediabetes and obesity? *Reprod Toxicol.* 31, 297-301.
- Slotkin, T. A., Card, J., Seidler, F. J., 2012. Chlorpyrifos developmental neurotoxicity: interaction with glucocorticoids in PC12 cells. *Neurotoxicol Teratol.* 34, 505-12.
- Svingen, T., Ramhoj, L., Mandrup, K., Christiansen, S., Axelstad, M., Vinggaard, A. M., Hass, U., 2018. Effects on metabolic parameters in young rats born with low birth weight after exposure to a mixture of pesticides. *Sci Rep.* 8, 305.
- Tinggaard, J., Wohlfahrt-Veje, C., Husby, S., Christiansen, L., Skakkebaek, N. E., Jensen, T. K., Grandjean, P., Main, K. M., Andersen, H. R., 2016. Prenatal pesticide exposure and PON1 genotype associated with adolescent body fat distribution evaluated by dual X-ray absorptiometry (DXA). *Andrology.* 4, 735-44.

- Wang, M., Lang, X., Zou, L., Huang, S., Xu, Z., 2011. Four genetic polymorphisms of paraoxonase gene and risk of coronary heart disease: a meta-analysis based on 88 case-control studies. *Atherosclerosis*. 214, 377-85.
- Waye, A., Trudeau, V. L., 2011. Neuroendocrine disruption: more than hormones are upset. *J Toxicol Environ Health B Crit Rev*. 14, 270-91.
- Weykamp, C., 2013. HbA1c: a review of analytical and clinical aspects. *Ann Lab Med*. 33, 393-400.
- WHO, 2011. Use of glycated haemoglobin (HbA1c) in diagnosis of diabetes mellitus: abbreviated report of a WHO consultation.
- Wohlfahrt-Veje, C., Main, K. M., Schmidt, I. M., Boas, M., Jensen, T. K., Grandjean, P., Skakkebaek, N. E., Andersen, H. R., 2011. Lower birth weight and increased body fat at school age in children prenatally exposed to modern pesticides: a prospective study. *Environ Health*. 10, 79.
- Xiao, X., Clark, J. M., Park, Y., 2017. Potential contribution of insecticide exposure and development of obesity and type 2 diabetes. *Food Chem Toxicol*. 105, 456-474.
- Yeste, D., Vendrell, J., Tomasini, R., Broch, M., Gussinye, M., Megia, A., Carrascosa, A., 2007. Interleukin-6 in obese children and adolescents with and without glucose intolerance. *Diabetes Care*. 30, 1892-4.
- Zhang, J., Liu, R., Gan, J., Liu, J., Liu, W., 2016. Endocrine-Disrupting Effects of Pesticides through Interference with Human Glucocorticoid Receptor. *Environ Sci Technol*. 50, 435-43.

Table1. Characteristics (N (%) or mean (95% CI)) of 141 children whose mothers were occupationally exposed or unexposed to pesticides during early pregnancy

	Unexposed (N=53)	Exposed (N=88)	p-value ^a
Sex (girls)	23 (43)	45 (51)	0.39
Maternal smoking in pregnancy (yes)	11 (21)	19 (22)	1.00
missing		3	
SES ^b	21/23/9 (40/43/17)	17/49/20 (20/57/23)	0.04
missing		2	
WGA (%)	2.7 (-1.3; 6.6)	-1.3 (-4.2; 1.6)	0.10
Child characteristics at follow-up ^c			
Age (years)	12.8 (12.3; 13.2)	13.2 (12.9; 13.5)	0.08
Tanner stage (1/2/3/4/5) ^d	7/10/10/13/13 (13/19/19/24.5/24.5)	4/15/21/24/24 (5/17/24/27/27)	0.45
Girls in puberty ^c	22 (96)	44 (98)	1.00
Boys in puberty ^c	24 (80)	40 (93)	0.15
Height (cm)	160.2 (157.1; 163.3)	160.7 (158.6; 162.9)	0.76
Height SDS ^f	0.33 (0.01; 0.66)	0.13 (-0.11; 0.37)	0.31
Weight (kg)	49.4 (46.0; 52.7)	53.8 (50.9; 56.7)	0.05
Weight SDS ^f	0.25 (-0.08; 0.57)	0.53 (0.27; 0.79)	0.18
Waist circumference (cm)	67.0 (64.6; 69.5)	70.0 (68.0; 72.0)	0.06
Body fat% (skinfolds)	19.2 (16.7; 21.8)	23.2 (21.2; 25.2)	0.02
BMI (kg/m ²)	19.1 (18.1; 20.2)	20.6 (19.8; 21.4)	0.02
BMI SDS ^f	0.06 (-0.29; 0.41)	0.56 (0.32; 0.81)	0.02
Systolic blood pressure (mmHg)	109 (107; 111)	112 (110; 115)	0.07
Diastolic blood pressure (mmHg)	55 (54; 57)	57 (56; 58)	0.09
Child PON1 192 QQ/QR or RR	26/25 (51/49)	46/40 (53.5/46.5)	0.86
missing	2	2	
Maternal PON1 QQ/QR or RR	13/21 (38/62)	36/33 (52/48)	0.21
missing	19	19	

^aDifferences between groups were tested using one-way ANOVA (continuous data) or Fisher's Exact Test (categorical data with two categories) or Likelihood Ratio (categorical data with more than two categories). ^b SES: Socioeconomic status (social class 1-3/4/5); ^cData obtained at that examination where the child delivered a blood sample for biomarker analyses; ^d Highest of Tanner

Pubic Hair stage, Tanner Breast stage (girls) and/or Tanner Genital stage (boys); ^e Tanner Stage > 1;

^fSDS: age and sex specific Standard Deviation Score.

Table 2. HbA1c and serum concentrations of metabolic and inflammatory markers in children whose mothers were occupationally exposed or unexposed to pesticides in early pregnancy. Results are presented as medians (5th - 95th percentiles)

	Unexposed	Exposed	p-value ^a
N	53	88	
HbA1c, mmol/mol	31.0 (25.2 - 37.8)	33.0 (28.0 - 38.0)	0.004
Leptin, pg/mL	6632 (715 - 44120)	11967 (1495 - 68933)	0.02
Adiponectin, ng/mL	9626 (2347 - 24031)	7102 (1942 - 22651)	0.03
Leptin/adiponectin ratio (LAR)	0.48 (0.07 - 6.44)	1.69 (0.15 - 17.02)	0.005
IGF-1, ng/mL	316.3 (148.6 - 477.6)	313.3 (132.6 - 502.7)	0.62
TNF- α , pg/mL	1.61 (1.01 - 2.68)	1.53 (0.94 - 2.10)	0.10
IL-6, pg/mL	0.38 (0.14 - 2.38)	0.46 (0.17 - 2.06)	0.14
hs-CRP, mg/L	0.20 (0.00 - 3.28)	0.20 (0.00 - 5.44)	0.98
Only samples from over-night fasting (N)	42	69	
Insulin, pmol/L	44.9 (16.0 – 117.4)	42.9 (17.0 - 150.9)	0.76
C-peptid, pmol/L	378.1 (225.8 – 795.1)	363.4 (228.6 - 868.6)	0.68
Insulin/c-peptide molar ratio	0.11 (0.06 – 0.33)	0.11 (0.07 - 0.17)	0.66
PAI-1, pmol/mL	5467 (3499 - 7948)	5959 (3783 - 9587)	0.07
Cholesterol, mmol/L	4.15 (2.90 – 5.66)	4.00 (2.60 – 5.20)	0.26
HDL, mmol/L	1.50 (1.08 – 2.15)	1.49 (0.87 – 2.29)	0.31
LDL, mmol/L	2.26 (1.10 – 3.37)	2.13 (1.30 – 3.26)	0.28
TG, mmol/L	0.69 (0.40 – 1.80)	0.71 (0.39 – 1.54)	0.96

^aDifferences between groups were tested using Mann-Whitney U Test.

Table 3. Adjusted^a relative differences (β) in percent with 95 % confidence intervals (95% CI) of HbA1c and related metabolic markers between children whose mothers were occupationally exposed to pesticides in early pregnancy and children of unexposed mothers

	β (95% CI)			p_{int}^c
	All children	Girls	Boys	
$N_{\text{unexposed}}/N_{\text{exposed}}$	53/88	23/45	30/43	
HbA1c, mmol/mol	5.0 (1.8; 8.2)**	6.2 (1.6; 11.1)**	3.9 (-0.3; 8.2)	0.47
Leptin, pg/mL	39.4 (-3.3; 101.0)	60.4 (-6.1; 174.0)	23.9 (-24.4; 102.9)	0.48
Adiponectin, ng/mL	-15.5 (-32.4; 5.7)	-7.7 (-33.5; 28.0)	-21.6 (-42.0; 6.1)	0.47
Leptin/adiponectin ratio (LAR)	65.0 (4.4; 160.9)*	73.8 (-11.3; 240.4)	57.9 (-15.0; 193.3)	0.83
IGF-1, ng/mL	-4.7 (-15.9; 8.0)	-6.3 (-22.2; 12.9)	-3.4 (-18.3; 14.2)	0.81
TNF- α , pg/mL	-2.8 (-9.9; 4.9)	2.4 (-8.3; 14.5)	-7.0 (-16.1; 2.9)	0.20
IL-6, pg/mL	17.6 (-10.1; 53.9)	21.1 (-18.3; 79.7)	14.7 (-20.2; 65.0)	0.84
OR ^b for hs-CRP above the median	1.1 (0.5; 2.2)	0.9 (0.3; 3.0)	1.2 (0.4; 3.2)	0.80
Samples from over-night fasting				
$N_{\text{unexposed}}/N_{\text{exposed}}$	42/69	18/37	24/32	
Insulin, pmol/L	-15.0 (-33.3; 8.3)	-22.5 (-45.3; 9.7)	-7.7 (-33.6; 28.4)	0.46
C-peptid, pmol/L	-5.2 (-18.3; 9.9)	-0.6 (-19.7; 23.0)	-9.2 (-25.8; 11.1)	0.54
PAI-1, pmol/mL	8.2 (-2.3; 19.7)	17.5 (1.6; 35.7)*	0.5 (-12.4; 15.2)	0.12
Cholesterol, mmol/L	-3.7 (-11.1; 4.4)	-1.8 (-12.5; 10.2)	-5.3 (-15.1; 5.6)	0.64
HDL, mmol/L	-5.5 (-14.0; 3.8)	-8.0 (-19.6; 5.3)	-3.3 (-14.9; 9.9)	0.59
LDL, mmol/L	-3.1 (-14.8; 10.3)	-1.2 (-17.9; 18.9)	-4.7 (-20.1; 13.7)	0.78
TG, mmol/L	1.4 (-12.9; 18.2)	11.4 (-10.4; 38.5)	-6.7 (-24.1; 14.7)	0.28

^aAdjusted for sex (all children), age, puberty (y/n) and SES, ^bodds ratio (OR) for having hs-CRP values above the median of 0.2 mg/L among exposed children compared to unexposed children, ^c p -value for exposure x sex interaction, * $p \leq 0.05$, ** $p \leq 0.01$

