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Prenatal pesticide exposure and *PON1* genotype associated with adolescent body fat distribution evaluated by dual X-ray absorptiometry (DXA).

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Abstract

Background Many modern pesticides have endocrine disrupting abilities and early life exposure may affect growth and disease risk later in life. Previously, we reported associations between prenatal pesticide exposure and higher childhood body fat content measured by anthropometry. The associations were affected by child *PON1* Q192R genotype.

Objective We aimed to study whether prenatal pesticide exposure was still associated with body fat content and distribution in the children at puberty and the potential impact of both maternal and child *PON1* Q192R genotype.

Methods A prospective cohort study of 247 children born by occupationally exposed or unexposed women (greenhouse workers and controls). Two follow-up examinations (age 10-15 and 11-16 years) included simple anthropometry, skinfold measurements, pubertal staging and blood sampling. Total and regional fat% was determined by dual X-ray absorptiometry (DXA) at age 10-15.

Results Prenatal pesticide exposure was associated with increased total, android and gynoid fat% (DXA) at age 10-15 years after adjustment for sex, socio-economic status and puberty (all0.5 standard deviation score (SDS) $p<0.05$). Stratified by sex, the associations were significant in girls (total fat: $\beta=0.7$ SDS, android-gynoid ratio: $\beta=0.1$, both $p<0.05$), but not in boys. Carrying the R-allele (child or mother, separately, or both) augmented the differences between exposed and unexposed children (total fat: $\beta=1.0$ SDS, $\beta=0.8$ SDS, $p<0.05$, respectively and $\beta=1.2$ SDS, $p<0.01$). No exposure related differences were found if either the child or mother had the QQ wildtype. At age 11-16, exposed children tended to have a higher total fat% estimated by skinfolds than unexposed children ($p=0.06$). No significant associations between prenatal exposure and BMI or waist circumference were found.

Conclusion Prenatal pesticide exposure was associated with higher adolescent body fat content, including android fat deposition, independent of puberty. Girls appeared more susceptible than boys. Furthermore, the association depended on maternal and child *PON1* Q192R genotype.

Introduction

Endocrine disrupting chemicals (EDCs), including some pesticides, are chemicals that can interfere with the endocrine system. Some EDCs may affect endocrine pathways responsible for adipose tissue differentiation (adipogenesis) or endocrine function of adipocytes (secretion of adipocytokines that are key regulators of energy balance and insulin sensitivity) (Janesick *et al,* 2011). Early-life exposure to such chemicals may alter metabolic programming of adipose tissue with permanent endocrine dysfunction and increased risk of metabolic diseases, including overweight, later in life (Perera *et al,* 2011;Stel *et al,* 2015;Tang-Peronard *et al,* 2011). Environmental EDC exposure therefore possibly contributes to the pathogenesis of the obesity epidemic that has emerged during the recent decades. The molecular mechanisms by which some pesticides may act as obesogens are not fully understood. Several pesticides have estrogenic or anti-androgenic properties *in vitro* (Andersen *et al,* 2002;Orton *et al,* 2011) which may affect fat distribution (Palmer *et al,* 2015). Besides, some pesticides have been found *in vitro* to potentiate gene expression of peroxisome proliferator-activated receptor-gamma (PPARγ) and activate for instance the glucocorticoid receptor (Kim *et al,* 2014;Sargis *et al,* 2010), both being important regulators of adipose tissue differentiation and function. In experimental studies, exposure of rats to otherwise subtoxic dose levels of organophosphate insecticides during development caused abnormalities in lipid and glucose metabolism, prediabetes and excess weight gain (Lassiter *et al,* 2008;Slotkin, 2011). In human studies, higher child- and adulthood body mass index (BMI) has been associated with early-life exposure to persistent organochlorine pesticides such as DDT and hexachlorobenzene (Karmaus *et al,* 2009;Smink *et al,* 2008;Valvi *et al,* 2012) but potential associations between exposure to modern pesticides and adipose tissue accumulation has not been examined.

We conducted a prospective cohort study including children whose mothers were employed in greenhouse horticulture with exposure to pesticides prior to recognition of pregnancy and children of occupationally unexposed mothers. From this cohort we have previously reported associations between pesticide exposure in the first trimester of pregnancy and lower birth weight followed by higher BMI Z-score and body fat percentage calculated from skinfolds at school age (Wohlfahrt-Veje *et al,* 2011). These findings are in accordance with several other studies reporting an association between prenatal exposure to modern pesticides and decreased birth weight, although

not all (Guo *et al,* 2014;Hanke *et al,* 2003;Jurewicz *et al,* 2005;Mayhoub *et al,* 2014;Rauch *et al,* 2012;Sagiv *et al,* 2007;Whyatt *et al,* 2004;Wolff *et al,* 2007). Furthermore, low birthweight has been found to be associated with increased risk of childhood overweight, in particular if followed by early catch-up growth (Baird *et al,* 2005;Monteiro *et al,* 2005;Ong *et al,* 2006;Stettler *et al,* 2010).

We have also reported that the associations between prenatal pesticide exposure and body fat content were found in those children who had a common genetic variant, a glutamine (Q) to arginine (R) substitution, at position 192 in the coding region of the *PON1* gene (Andersen *et al,* 2012). Exposed children carrying the minor allele (R) had higher abdominal circumference, BMI Z-score and body fat percentage calculated from skinfolds at school age than unexposed children while none of these variables were significantly affected in children with the *PON1* 192QQ wildtype. *PON1* codes for paraoxonase 1 (PON1), a multifunctional high density lipoprotein (HDL)-associated enzyme, that has esterase, lactonase and antioxidant activities (Macharia *et al,* 2012;Gugliucci *et al,* 2015). PON1 hydrolyses a range of substrates including the toxic oxon derivatives of some organophosphate insecticides and also prevents lipid oxidation (Mackness *et al,* 2002;Richter *et al,* 2010). PON1 is thereby linked to toxicity of some pesticides and atherosclerotic disease.

In this study we have followed the children during puberty to investigate whether the association between prenatal pesticide exposure and body fat content persists into adolescent age. To improve the validity of our data on body fat content, we supplemented anthropometry with dual X-ray absorptiometry (DXA), a highly accurate measure of total and regional fat percentage (%) (Atherton *et al,* 2013). Furthermore, we aim to investigate the impact of both maternal and child *PON1* Q192R polymorphism on this association.

Materials and Methods

Study population

This is a prospective cohort study including 247 children born between 1996 and 2001, of whom 203 children had their first clinical examination at 3 months of age and 44 additional age-matched controls were included when the children were between 6 to 11 years of age (Fig. 1). The cohort

has previously been described in detail (Andersen *et al,* 2008;Wohlfahrt-Veje *et al,* 2011). In brief, we recruited pregnant women employed in greenhouses in Funen, Denmark and referred to the Department of Occupational and Environmental Medicine at Odense University Hospital for risk assessment of their working conditions and guidance for safe work practises during pregnancy. Pesticide exposure assessment was made independently by two toxicologists with special expertise in working conditions in greenhouse horticultures. Information was obtained from detailed questionnaire-assisted interview of the pregnant women and from telephone interview of the employers. Some of the women were exposed to mixtures of pesticides in the first trimester of their pregnancy before preventive measures were taken. The women were classified as occupationally exposed if pesticides were applied in the working area more than once a month and the women handled treated plants within one 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. The active compounds most frequently used in the working area were the insecticides, deltamethrin, dichlorvos, dimethoate chlorpyrifos and endosulfan; the fungicides captan, chlorothalonil, fenarimol, fosetyl-aluminium and iprodion and the growth regulators daminozid, paclobutrazol, chlormequat chloride and ethephon. However, more than 100 different pesticide formulations were used and the women were exposed to a variety of different mixtures of these pesticides. A detailed list of all the pesticides used in the greenhouses is available from the corresponding author. Information on birth weight (BW), birth length (BL) and gestational age was obtained from the obstetric records. Weight-for-gestational age (WGA) was calculated according to Marsal (Marsal *et al,* 1996). Information on lifestyle factors (including maternal smoking during pregnancy) was obtained from a questionnaire administered at the first clinical examination of the child. Socioeconomic status (SES) was grouped into 1=high to 5=low based on parental education and occupation (Hansen, 1978).

Here we present data from follow-up examinations when the children were between 10 to 15 years of age (referred to as Puberty examination 1, performed late 2011 to early 2012) and between 11 to 16 years of age (referred to as Puberty examination 2, performed first half of 2013) (Fig. 1). At Puberty examination 1, a total of 243 children were eligible for participation (4 were lost to follow-up) and 163 accepted the invitation (participation rate 67 %). At Puberty examination 2, 229 children were eligible for participation (14 families were lost to follow-up) and 133 accepted the invitation (participation rate 58%). 128 of the 133 children participating in Puberty examination 2

also participated in Puberty examination 1. One examination sheet was lost from Puberty examination 2 leaving clinical data on 132 children.

Clinical examination

The clinical examination included measurement of height to the nearest mm using a portable Harpenden Stadiometer (Holtain Ltc, Crymych, United Kingdom), weight to the nearest 0.1 kg by electronic scale (SECA delta model 707, Hamburg, Germany), hip- and waist- circumference using a SECA measurement tape and measurement of skinfold thickness to the nearest mm (biceps, triceps, subscapularis, suprailiac) using a Harpenden calliper (John Bull, British Indicators LTD, Weybridge, UK). All measurements were standardised and done in triplet by the same trained paediatrician (JT), who was blinded to the exposure status. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Total body fat percentage was calculated from skinfolds by the Slaughter equation (Slaughter *et al*, 1988):
In girls: $(1.33(\text{triceps+subscapular}) - 0.013(\text{triceps+subscapular})^2 - 2.5)$ or $(0.546(\text{triceps+subscapular}) + 9.7)$ when sum of triceps and subscapular > 35 mm. In prepubertal boys: $(1.21(\text{triceps+subscapular}) - 0.008(\text{triceps+subscapular})^2 - 1.7)$, in pubertal boys: $(1.21(\text{triceps+subscapular}) - 0.008(\text{triceps+subscapular})^2 - 3.4)$, in postpubertal boys $(1.21(\text{triceps+subscapular}) - 0.008(\text{triceps+subscapular})^2 - 5.5)$ or $(0.783(\text{triceps+subscapular}) + 1.6)$ when sum of triceps and subscapular > 35 mm.
Pubertal stage was assessed by palpation and inspection according to Tanner and Marshall (Marshall *et al,* 1969;Marshall *et al,* 1970). In girls, pubertal onset was defined as Tanner stage B2 uni- or bilaterally and/or Tanner Stage PH2. In boys, pubertal onset was defined as Tanner stage G2 and/or Tanner stage PH2. Postpuberty was defined as Tanner stage B5/G5 and PH5. A combined Tanner stage was defined as the highest of B-, G- and/or PH-stage.
All clinical assessments were performed by a single trained investigator (JT). All examinations were performed blinded to information about prenatal pesticide exposure.

Dual X-ray Absorptiometry

A whole body DXA scan was performed (Lunar Prodigy, GE Healthcare, Madison, WI, USA) on the same day as the clinical examination at Puberty examination 1. All children participating in Puberty examination 1 underwent DXA. The children wore standardized light clothing. A single trained investigator visually checked all scans for positioning and corrected predefined body regions for analysis if necessary. Total and regional mass (g), fat mass (g) and fat percentage (%) was determined. Total fat% was expressed as total fat mass divided by total body mass. Android and gynoid fat% was expressed as android fat mass/android mass and gynoid fat mass/gynoid mass, respectively. Android/gynoid fat% ratio was calculated by simple division.

Laboratory analysis

Venous blood samples from children and mothers were collected in EDTA coated vials. After centrifugation at 2000 g for 10 min. at 20 °C, buffy coat was separated and stored at – 80 °C until analysis. For *PON1* genotyping, DNA was isolated from buffy coat samples and *PON1* genotyped as previously described by the Taqman-based allele discrimination using the ABI Prism 7700 Sequence Detection System (Andersen *et al*, 2012). Q192R (rs662) polymorphisms of the *PON1* gene have previously been determined for 141 children who participated in the follow-up between 6 to 11 years of age (Andersen *et al*, 2012). In this study Q192R (rs662) polymorphisms were determined for additional 40 children and 122 mothers. For 7 of the 163 children who underwent DXA scan blood samples for PON1 genotyping were not available.

Statistical analyses

Age and sex specific standard deviation scores (SDS) for height, weight, BMI, and total body fat% measured by DXA were calculated according to national references (Tinggaard *et al*, 2014;Wohlfahrt-Veje *et al*, 2014). Furthermore, age and sex specific SDS for android and gynoid fat% were calculated using data from 982 healthy Danish children (425 girls) aged 8-15 years as reference (Wohlfahrt-Veje *et al*, 2014). Within-population Z-scores for waist circumference and total body fat percentage calculated from skinfolds were calculated by a linear regression model with waist circumference or total body fat percentage calculated from skinfolds (both log10

transformed) as dependent and age and sex as independents. The standardized residuals from these analyses correspond to within-population Z-scores.

Differences between characteristics of the exposed and unexposed children were tested by Fishers exact test (dichotomous variables) or Likelihood Ratio (categorical variables with > two categories) and Mann-Whitney U test (continuous variables) because not all parameters were normally distributed.

Multiple linear regression analysis was used for association analyses between prenatal pesticide exposure and body composition outcomes. Interaction between exposure and sex or *PON1* Q192R polymorphism was examined by including an interaction term in the adjusted analysis. Presence of interaction was defined at $p < 0.2$ due to low statistical power for detection of interaction. Analyses were stratified by sex to examine effect modification by sex or if interaction was found. The following variables were included as confounders/covariates: sex, age at examination, SES (combining SES 1, 2 and 3 into one group evening the number of cases in each group and included as dummy variables using the largest group (SES 4) as reference), and pubertal development (yes/no or Tanner Stage 1 to 5). Maternal smoking in pregnancy (yes/no) was considered, but was not included because of significant correlation with SES (Chi-square, $p = 0.027$).

Due to the low frequency of minor allele homozygosity (RR), minor allele homozygosity (RR) and heterozygosity (QR) were combined (RR/QR). Sensitivity analyses excluding one boy with type I diabetes mellitus from the analysis did not affect the results.

All analyses were performed using IBM SPSS Statistics version 22.0. A p-value < 0.05 was considered statistically significant.

Ethics

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

Results

Population characteristics, anthropometric data and DXA measurements are presented in Table 1. At Puberty examination 1, exposed children had statistically significant higher BMI (SDS), waist circumference and total body fat% calculated from skinfolds (Z-score) and total, android and gynoid fat% (SDS) measured by DXA (all unadjusted). Furthermore, the android/gynoid ratio was higher in exposed children compared to unexposed children. At Puberty examination 2, exposed children had a higher total fat% calculated from skinfolds (Z-score) compared to unexposed children (unadjusted), whereas all other parameters showed similar trends as Puberty examination 1 but did not reach statistical significance. Median age, percentage of pubertal children and percentage of mothers having smoked during pregnancy were similar for exposed and unexposed children at both examinations (Table 1). However, WGA was lower in exposed children and SES was lower among parents of exposed children compared to unexposed.

After adjustment for relevant covariates, BMI (SDS) and total fat% calculated from skinfolds (Z-score) was higher in exposed children compared to unexposed children at Puberty examination 1 (Table 2). At Puberty examination 2, difference in skinfold total fat% was borderline significant (p=0.06) (Table 2). No differences in waist circumference (Z-score) at any of the two examinations were found. Although the interaction term between exposure and sex was not significant, analyses stratified by sex showed that associations between prenatal exposure and skinfold total fat% remained statistically significant in girls, but not boys. Likewise, BMI was higher in exposed girls compared to unexposed girls, though of borderline significance (p=0.06) (Table 2).

Total, android and gynoid fat% (crude values and SDS) determined by DXA was higher among exposed children compared to unexposed children (adjusted analyses) (Table 3). The variance in fat% was greater in girls compared to boys and interaction between exposure and sex was found for unstandardized outcomes (total fat%: p=0.16, android fat%: p=0.07 and android-gynoid ratio: p=0.06). When outcomes were age and sex standardized the variances were similar and the interaction term was no longer significant. However, in analyses stratified by sex, the associations between prenatal pesticide exposure and both crude and standardized outcomes remained significant in girls, but not in boys (Table 3). Furthermore, the andoid-gynoid ratio was higher in exposed girls compared to unexposed girls (Table 3). Including WGA as a covariate tended to increase the effect estimates, though not markedly (data not shown).

Heterogeneity in associations between DXA body fat percentage with child and maternal *PON1* Q192R polymorphism

The genotype frequencies for *PON1* Q192R in mothers (n=122) were: wildtype (QQ) 49 %, heterozygosity (QR) 46 % and homozygosity (RR) 5 %. The minor allele frequency was 28 %. The genotype frequencies for *PON1* Q192R in children were: QQ 52 %, QR 40 % and RR 8 %. In children, the minor allele frequency was 28 %.

PON1 Q192R genotype of the child or the mother was only associated with DXA fat% outcome measures when exposure was included in the analyses (data not shown). Furthermore, the effect of prenatal pesticide exposure depended on both maternal and child *PON1* Q192R genotype, except the effect of maternal *PON1* Q192R genotype on android/gynoid ratio (Table 4). Exposed children being carriers of the minor allele (RR/QR) or whose mothers were carriers of the minor allele (RR/QR) had significantly higher total, android and gynoid fat% measured by DXA compared to unexposed children with the same genotype (Table 4). Furthermore, prenatal pesticide exposure was associated with an increased android/gynoid ratio in exposed children compared to unexposed children with the RR/QR genotype (Table 4). The mean differences between exposed and unexposed children were greater if the children were carriers of the minor allele compared to the mothers being carriers (Table 4). No differences in body fat% between exposed and unexposed QQ children were seen (Table 4).

When child and maternal *PON1* QR/RR genotype was combined, the associations between exposure and fat% were potentiated (Fig. 2A-2D; total fat: β=1.2 SD [0.4-2.1], android fat: β=1.3 SD [0.5-2.1], gynoid fat: β=1.2 SD [0.3-2.2] and android-gynoid ratio: β= 0.24 [0.09-0.39]). The differences between exposed and unexposed children were not significant if just one of them carried the minor allele or if both were wildtype (Fig. 2).

Unexposed RR/QR children of RR/QR mothers had a lower fat% SDS compared to other unexposed children (Fig 2A-2D; total fat: β=0.8 SD [0.1-1.6], android fat: β=0.8 SD [0.1-1.5], gynoid fat: β=0.9 SD [0.1-1.6] and android-gynoid ratio: β=0.13 [0.01-0.26]). Conversely, exposed RR/QR children of RR/QR mothers had a higher android/gynoid ratio compared to other exposed children (β=0.11 [0.02-0.20]) (Fig. 2D).

Discussion with conclusions

Prenatal pesticide exposure was associated with an increase of body fat measured by DXA and skinfolds in pubertal children born by greenhouse workers who carried the minor allele of the *PON1* Q192R polymorphism. These findings are in agreement with our previous findings when the children were 6 to 11 years old and suggest a long-term effect on body composition. The increment in body fat percentage estimated by skinfolds was lower than observed in the same cohort of children when examined during childhood, which suggests an impact of puberty and age (Taylor *et al,* 2010). To our knowledge, this is the first study to describe total and regional fat percentage measured by DXA in pubertal children prenatally exposed to pesticides. Fat estimates based on DXA are more accurate than standard anthropometry (Atherton *et al,* 2013)which may explain why associations were more pronounced in DXA outcomes compared with simple anthropometry such as waist circumference.

The exposure related differences in body fat percentages were more pronounced in girls than boys, suggesting a sex-specific susceptibility to metabolic disturbances after prenatal pesticide exposure. Alternatively, the discrepancy between girls and boys could be related to the higher fat mass in girls by itself, more clearly demarcating effects of exposure. Similar observations have been reported from other human studies examining the association between prenatal or early exposure to persistent organochlorines such as polychlorinated biphenyls (PCBs) and DDT and childhood obesity (Gladen *et al,* 2000;Hertz-Picciotto *et al,* 2005;Valvi *et al,* 2012)). We previously reported earlier breast development among the exposed girls in this cohort compared to unexposed girls (Wohlfahrt-Veje *et al,* 2012). Thus, prenatal pesticide exposure may affect both fat mass and pubertal development which are known to be interlinked. However, including puberty as a confounder in the sex stratified analyses did not substantially alter the effect estimates in girls (Table 3).

The estimated mean total fat% in exposed girls/boys was 28.7%/21.4% compared to 22.5%/20.1% in unexposed girls/boys, respectively. In comparison, average fat percentage in this age group is 22% for girls and 17% for boys (Wohlfahrt-Veje *et al,* 2014). We hypothesize that this increase in body fat may be clinically relevant in girls as accumulation of adipose tissue is accompanied by dysfunctional adipocytes and may have detrimental metabolic implications (Ali *et al,* 2013). Furthermore, prenatal exposure tended to be associated with a larger degree of abdominal (android) than peripheral (gynoid) fat accumulation, reflected by the higher android/gynoid ratio. Abdominal

adiposity is associated with an increased risk of metabolic and cardiovascular disease independently of BMI both in adults and children (Gishti *et al,* 2015;Oliveros *et al,* 2014). Though peripheral fat, which may be protective for metabolic disease, was also increased in exposed children, the android/gynoid ratio was shifted towards a metabolically unhealthy profile in girls (Gishti *et al,* 2015). Furthermore, android/gynoid ratio may be a better predictor of metabolic and cardiovascular disease risk in children than android fat% itself, emphasising the importance of assessing fat distribution (Samsell *et al,* 2014;Zong *et al,* 2015). Therefore these findings are of particular concern.

As previously reported (Wohlfahrt-Veje *et al,* 2011), WGA was lower in exposed children than unexposed children and as low WGA, especially followed by rapid infancy growth, is associated with childhood obesity (Stettler *et al,* 2010;Ong *et al,* 2006) the effect of prenatal pesticide exposure may be mediated by this. However, including WGA in the analyses tended to increase rather than decrease the estimates of the association between prenatal pesticide exposure and pubertal fat content. This supports an independent effect of exposure on body fat accumulation.

We have previously reported a gene-environment interaction between child *PON1* Q192R genotype and prenatal pesticide exposure on body fat content estimated by anthropometry among the children at 6 to 11 years of age (Andersen *et al,* 2012). This pubertal follow-up confirms the gene-environment interaction, since body fat accumulation was only enhanced if either the child or the mother or both carried the *PON1 192*R-allele. Hence, the differential effects on body fat content related to child *PON1* gene heterogeneities persisted into puberty. In addition, we found that also the maternal *PON1* Q192R genotype affected the associations. Mothers carrying the R-allele may detoxify some pesticides more slowly and have reduced anti-oxidative capacity to protect against lipid oxidation, resulting in a more unhealthy metabolic profile (Aviram *et al,* 2000;Costa *et al,* 2015;Macharia *et al,* 2012). This might result in a more adverse intrauterine environment affecting metabolic programming. Furthermore, although the expression of the *PON1* gene is low in new-borns and probably also in fetal life, the fetal *PON1* genotype may be associated with epigenetic changes, such as DNA-methylation in the *PON1* gene, thereby altering *PON1* expression and related risk profile later in life (Huen *et al,* 2015;Liu *et al,* 2013) Our findings of a larger effect of prenatal exposure if both the child and the mother carried the R-allele suggests for an additive effect.

We found that unexposed children had a significantly lower total and regional body fat% if both the child and the mother carried the minor allele compared to other unexposed children. This finding accounts for part of the larger difference in body fat% between exposed and unexposed QR/RR children of QR/RR mothers. However, this may be a chance finding due to the limited number of children in each subgroup. Therefore, these findings should be interpreted with caution and needs to be replicated in a larger cohort study.

Among women working in floricultures in Mexico, those with the *PON1* 192RR genotype had higher risk of miscarriage (Blanco-Munoz *et al*, 2013) and having children with low birth weight than women with the QQ or QR genotype (Moreno-Banda *et al*, 2009). In contrast, maternal *PON1* Q192R genotype did not affect the association between maternal organophosphate concentration during pregnancy and child birth outcome measures in the Chamacos Study, nor did it affect the PON1 activity in the child. In this cohort, child PON1 wildtype was associated with childhood obesity at age 2 years, but possible effects of pesticide exposure was not included (Huen *et al,* 2013). The effect of *PON1* genotype is complex. *PON1* Q192R genotype contributes only partly to the variance in PON1 activity, PON1 activity varies with age (Harley *et al*, 2011;Huen *et al,* 2009;Huen *et al,* 2010) and distribution of *PON1* Q192R polymorphism varies with ethnicity (Huen *et al*, 2013). These factors all complicate study comparison along with differences in type, timing and level of exposure and likely account for some of the discrepant results. In our study, the mothers were exposed to a variety of different pesticides including organophosphates and several fungicides with known endocrine disrupting properties. Due to the complex exposure setting it was not possible to identify specific pesticides as responsible for the effects. However, mixed exposure is a real-world situation and the effects may very well be related to combined exposures to several pesticides. PON1 is known to detoxify toxic oxon derivatives of some organophosphate insecticides and organophosphates were used occasionally in the working areas for most of the exposed mothers. However, only very few mothers had been involved directly in their application. Whether PON1 can detoxify other types of pesticides than organophosphates has, to our knowledge, not yet been investigated. The mothers were only occupationally exposed to pesticides until 8 to 12 weeks of gestation. The first weeks of gestation is a period of high vulnerability to interference with epigenetic reprogramming and exposure may have detrimental health effects for the unborn child (Perera *et al*, 2011). However, epigenetic changes occurs throughout life (Perera *et al*, 2011) and may counterbalance part of the adverse effects of prenatal exposure.

Limitations to this cohort study include the relatively small sample size and potential selection bias introduced by loss of children to follow-up and inclusion of additional controls. These limitations have been addressed in previous publication (Andersen *et al,* 2012;Wohlfahrt-Veje *et al,* 2011). Although the association between prenatal exposure and outcome measurements are consistent, use of exposure biomarkers would have strengthened our findings. However, our study design did not allow bio-monitoring of pesticide exposure in the mothers and the exposure classification encompassed more than 100 pesticides used in different mixtures. The blinded exposure classification, the blinded clinical examinations and the longitudinal design minimize the possible impact of exposure misclassification and bias. Diet and physical activity are relevant covariates, which we were not able to adjust for. However, we include SES in our analyses which may partly adjust for these factors because SES and lifestyle are correlated. Other possible confounders are maternal pre-pregnancy BMI and gestational weight gain, which this study lacks information on. Furthermore, some of the mothers resumed working in greenhouses after maternity leave and take-home exposure in these cases cannot be excluded. However, we do not have valid information for the relevant period allowing us to adjust for this.

In conclusion, prenatal exposure to non-persistent pesticides was associated with long-term increase in body fat percentage, especially of android fat, during puberty. Girls seemed more susceptible than boys and the effect of prenatal exposure also depended on maternal and child *PON1* Q192R genotype, indicating complex interactions between sex, prenatal environment and genetics.

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Disclosures

The authors have nothing to disclose

Authors' contributions

Jeanette Tinggaard: writing of the manuscript, literature search, study design, data collection, data analyses, data interpretation, tables and figures.

Christine Wohlfahrt-Veje, Steffen Husby, Lene Christiansen, Niels E Skakkebæk, Tina K Jensen, Philippe Grandjean: Study design, data interpretation, data analyses, writing of manuscript. Lene Christiansen also contributed in data collection.

Helle R Andersen: Supervision, study design, data collection, data interpretation, data analyses, writing of manuscript.

Katharina M Main: Supervision, study design, data interpretation, data analyses, writing of manuscript.

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Table 1 Population characteristics, anthropometric data and DXA data in children participating in Puberty examination 1 and 2.

	Puberty examination 1		**Puberty examination 2**	
	Unexposed (n=62)	**Exposed (n=101)**	**Unexposed (n=50)**	**Exposed (n=83)**
Sex (girls)	26 (42)	46 (45)	21 (42)	39 (47)
Smoking in pregnancy (yes)	11 (18.0)	25 (24.8)	6 (12.0)	18 (22.0)
SES[#] Missing SES	24/25/13 (39/40/21)	19/57/24 (19/57/24)* 1(1)	21/19/10 (42/38/20)	15/48/18 (19/59/22)* 2 (2)
Tanner Stage[##] Puberty (yes) [###] Missing Tanner Stage/Puberty	12/10/15/10/11 (21/17/26/17/19) 46 (79.3) 4 (6)	14/20/19/19/21 (15/22/20/20/23) 79 (84.8) 8 (8)	0/5/14/11/14 (0/11/32/25/32) 50 (100.0) 6 (12)	1/7/20/22/25 (1/10/27/29/33) 74 (98.7) 8 (10)
Age (years)	12.3 (10.2-14.7)	12.5 (10.6-14.2)	13.6 (11.5-16.1)	13.9 (12.0-15.8)
Height (cm) Height (SDS)	156.1 (137.0-176.7) 0.45 (-1.99-2.20)	157.5 (139.2-173.0) 0.17 (-2.04-2.24)	162.7 (145.3-178.0) 0.11 (-2.08-1.69)	163.8 (145.5-180.2) 0.10 (-1.94-2.01)
Weight (kg) Weight (SDS)	46.5 (29.9-64.1) 0.22 (-1.89-2.11)	48.0 (33.5-76.9) 0.53 (-1.78-2.72)*	52.8 (33.3-86.8) 0.11 (-1.83-2.39)	54.5 (37.4-85.1) 0.50 (-1.91-2.65)
BMI (kg/cm^2) BMI (SDS)	17.6 (14.5-25.9) -0.13 (-1.76-2.38)	19.7 (15.5-27.3)** 0.64 (-1.47-2.39)**	18.9 (15.2-30.9) 0.02 (-1.57-2.96)	20.6 (16.3-28.2) 0.50 (-1.36-2.45)
WGA (%)	4.0 (-18.3-25.0)	-3.2 (-27.1-19.1)*	4.9 (-18.0-32.2)	-3.8 (-23.0-19.1)*
Waist circum.(cm) Waist circum.(Z-score)	64.7 (56.3-83.5) -0.48 (-1.39-1.58)	67.6 (56.4-85.1)** 0.01 (-1.32-2.01)**	67.2 (58.0-98.3) -0.37 (-1.20-2.47)	69.0 (59.2-90.1) -0.11 (-1.32-1.99)
Skinfolds (mm) triceps subscapular suprailiac biceps	10.9 (5.5-21.5) 7.1 (4.3-20.5) 8.6 (4.4-26.3) 10.3 (5.2-20.6)	12.9 (7.5-24.7)** 9.5 (5.4-27.6)** 11.5 (5.1-29.9)** 12.3 (6.9-21.7)**	10.8 (5.6-28.5) 7.6 (4.6-35.3) 9.4 (3.9-41.0) 9.3 (3.6-29.4)	13.6 (6.8-28.9) 11.0 (5.7-36.1)** 14.0 (4.7-37.2) 10.1 (4.2-25.2)
Skinfolds total fat% Skinfolds total fat% (Z-score) Missing skinfolds total fat%	17.0 (9.9-34.2) -0.44 (-1.92 (1.81) 3 (5)	20.6 (12.6-39.0)** 0.02 (-0.18-1.82)** 6 (6)	17.8 (8.9-51.6) -0.50 (-1.63-1.96) 5 (10)	22.7 (11.7-46.4)* 0.05 (-1.25-1.78)* 7 (8)
DXA				
Total fat % Total fat % (SDS)	20.5 (8.1-39.1) 0.16 (-1.95-1.99)	26.7 (11.1-43.1)** 0.71 (-0.95-2.33)**	NI	NI
Android fat % Android fat % (SDS)	20.7 (7.6-46.9) 0.12 (-1.50-1.91)	29.0 (10.9-51.3)** 0.76 (-0.77-2.17)**	NI	NI

Gynoid fat % Gynoid fat % (SDS)	32.8 (12.6-49-1) 0.18 (-2.04-2.15)	38.1 (19.3-51.9)** 0.74 (-0.97-2.51)**	NI	NI
Android/gynoid ratio	0.66 (0.48-1.03	0.79 (0.48-1.07)*	NI	NI

Values are presented as median (5-95 percentiles) for continuous variables and as n (%) for categorical variables. Differences between unexposed and exposed children are tested using Mann-Whitney U-test for continuous variables and Fishers exact test (dichotomous variables) or Likelihood Ratio (categorical variables with > 2 categories).
* p-value < 0.05
** p-value < 0.01
[#] SES: Socioeconomic status (social class 1-3/4/5)
[##] Highest of Tanner Pubic Hair stage, Tanner Breast stage (girls) and/or Tanner Genital stage (boys)
[###] Tanner Stage > 1
SDS: Standard Deviation Score
NI: not investiged

Table 2 Associations between prenatal pesticide exposure and anthropometric measurements in Puberty examination 1 and 2. β represents adjusted difference (95% CI) in outcome variable between exposed and unexposed children.

	Puberty examination 1				**Puberty examination 2**			
n=exposed/ n=uexposed	**All children 101/61**		**Girls 46/26**	**Boys 55/36**	**All children 82/49**		**Girls 39/21**	**Boys 44/29**
	β (95% CI)[#]	**p-value interaction exposure and sex**	**β (95% CI)[#]**	**β (95% CI)[#]**	**β(95% CI)[#]**	**p-value interaction exposure and sex**	**β (95% CI)[#]**	**β (95% CI)[#]**
BMI SDS	0.4 (0.0-0.9)*	0.69	0.6 (0.0-1.1)	0.4 (-0.2-1.0)	0.3 (-0.2-0.7)	0.66	0.2 (-0.4-0.8)	0.3 (-0.4-1.0)
Waist circum. Z-score	0.3 (-0.1-0.6)	0.31	0.4 (-0.1-0.9)	0.1 (-0.3-0.6)	0.1 (-0.3-0.5)	0.92	0.1 (-0.4-0.7)	0.2 (-0.4-0.7)
Skinfolds total fat% Z-score	0.5 (0.1-0.8)**	0.59	0.6 (0.2-1.0)**	0.4 (-0.1-0.9)	0.4 (0.0-0.7)	0.79	0.4 (-0.1-0.8)	0.4 (-0.2-1.0)

[#] adjusted for sex (all children), Tanner Stage and SES
* p-value < 0.05
** p-value < 0.01

Table 3 Associations between prenatal pesticide exposure and fat% measured by DXA in Puberty examination 1. β represent adjusted mean differences (95% CI) in outcome variables between exposed and unexposed children.

n=exposed/n=uexposed	**All children 101/62**		**Girls 46/26**	**Boys 55/36**
	β (95% CI) [#]	**P-value for interaction exposure and sex**	**β (95% CI)** [#]	**β (95% CI)** [#]
Total fat %	3.80 (0.76-6.84)*	0.16	6.18 (1.82-10.54)**	1.33 (-3.15-5.82)
Total fat % SDS	0.53 (0.17-0.89)**	0.43	0.73 (0.20-1.27)**	0.39 (-0.11-0.89)
Android fat %	4.50 (0.39-8.62)*	0.07	8.66 (2.74-14.59)**	0.56 (-5.53-6.66)
Android fat % SDS	0.45 (0.10-0.79)*	0.29	0.67 (0.16-1.18)*	0.28 (-0.20-0.75)
Gynoid fat%	4.11 (1.05-7.17)**	0.38	5.49 (1.58-9.40)**	2.19 (-2.63-6.99)
Gynoid fat % SDS	0.54 (0.16-0.92)**	0.47	0.75 (0.19-1.32)**	0.40 (-0.13-0.93)
Android/Gynoid ratio	0.04 (-0-02-0.10)	0.06	0.11 (0.02-0.20)*	-0.01 (-0.10-0.08)

[#]Absolute values adjusted for sex (all children), age, SES and puberty (yes/no).

SDS adjusted for sex (all children), SES and puberty (yes/no).

*p-value <0.05

**p-value <0.01

Table 4 Associations between prenatal pesticide exposure and fat% measured by DXA stratified by child or maternal *PON1* Q192R genotype. β represent adjusted mean differences (95 % CI) in outcome variables between exposed and unexposed children.

[#]

	Child *PON1* Q192R genotype			**Maternal *PON1* Q192R genotype**		
n=exposed/n=unexposed	**QQ 52/29**	**RR/QR 47/28**		**QQ 43/17**	**RR/QR 38/24**	
	β (95% CI)[#]	**β (95% CI)[#]**	**P-value interaction[a]**	**β (95% CI)[#]**	**β (95% CI)[#]**	**P-value interaction[b]**
Total fat%	0.25 (-4.23-4.72)	8.12 (3.90-12.33)**	0.01	2.08 (-3.28-7.31)	7.86 (2.41-13.31)**	0.11
Total fat% SDS	0.11 (-0.41-0.64)	1.00 (0.48-1.50)**	0.02	0.33 (-0.25-0.91)	0.75 (0.11-1.39)*	0.40
Android fat%	1.24 (-7.32-4.84)	11.47 (5.78-17.16)**	<0.01	2.75 (-4.76-10.25)	9.72 (2.32-10.25)*	0.17
Android fat% SDS	0.00 (-0.51-0.50)	0.95 (0.48-1.43)**	<0.01	0.29 (-0.28-0.87)	0.71 (0.11-1.31)*	0.41
Gynoid fat%	0.56 (-3.70-4.83)	7.85 (3.28-12.42)**	0.02	1.80 (-3-34-6.94)	8.73 (3.16-14.30)**	0.06
Gynoid fat% SDS	0.12 (-0.42-0.66)	1.00 (0.44-1.56)*	0.03	0.26 (-0.35-0.87)	0.77 (0.10-1.44)*	0.34
Android/gynoid ratio	-0.05 (-0.14-0.05)	0.15 (0.06-0.23)**	<0.01	0.04 (-0.08-0.15)	0.09 (-0.02-0.20)	0.51

adjusted for age and sex (absolute values), SES and puberty (Y/N)

[a] Interaction between exposure and Child PON1 Q192R genotype

[b] Interaction between exposure and Maternal PON1 Q192R genotype

*p-value < 0.05, **p-value < 0.01

Figure 1 Flowchart of the cohort.

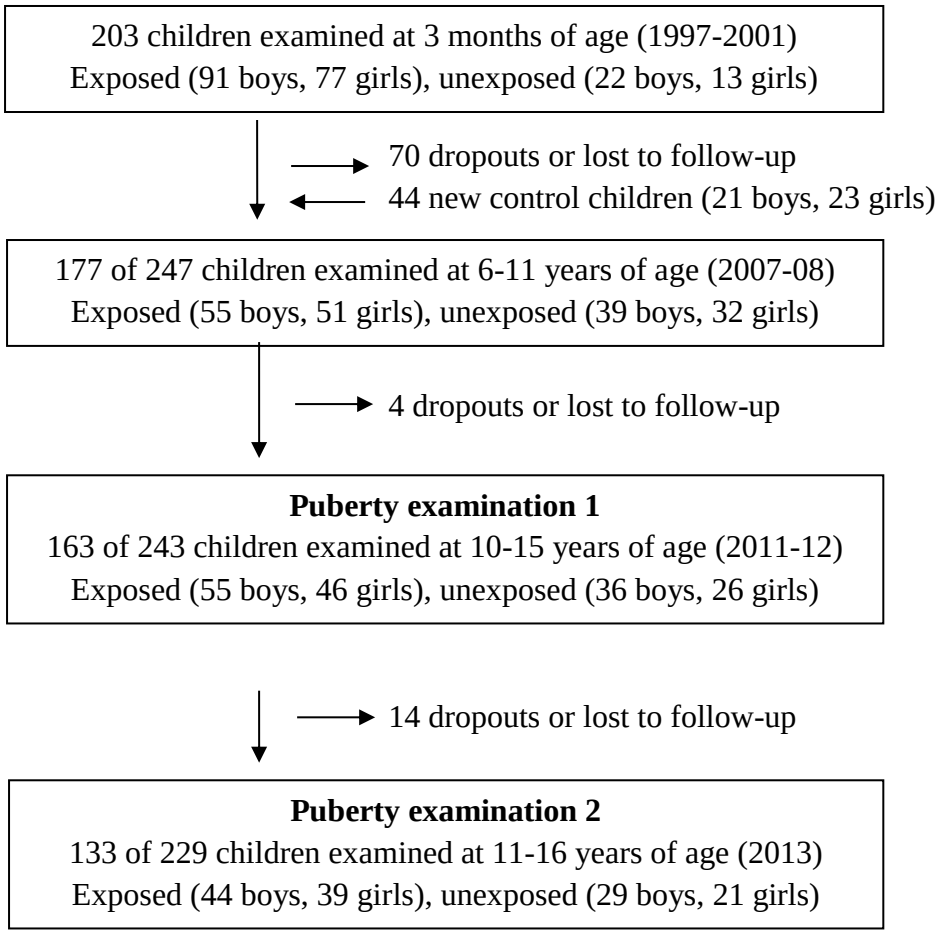

Figure 2 Estimated total body fat% SDS (A), android fat% SDS (B), gynoid fat% SDS (C) and android/gynoid ratio (D) measured by DXA in exposed children (black) and unexposed children (dotted black) stratified by combined maternal and child *PON1* Q192R genotype. SDS outcomes are adjusted for SES and puberty (yes/no) and android-gynoid ratio further adjusted for age and sex.. Open dots represent means, error bars 95% CI. Significant p-values between exposed and unexposed QR/RR children of QR/RR mothers are presented as well as significant p-values between exposed or unexposed QR/RR children and other exposed or unexposed children, respectively.