Introduction

The serum enzyme paraoxonase 1 (PON1) is a high density lipoprotein (HDL)-associated enzyme with antioxidant function [1]. It protects lipoproteins from oxidative modifications, and thus protects against the development of atherosclerosis [2,3]. This enzyme hydrolyzes many substrates, including organophosphate pesticides. A common polymorphism, PON1 Q192R, affects both the catalytic activity and the anti-atherogenic properties [2]. Hence, an association between the R-allele and the risk of developing cardiovascular disease (CVD) has been reported in case-control studies [2,9,10]. 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 [11]. Although the PON1 Q192R genotype seems to affect both the catalytic activity and the anti-atherogenic properties, a potential interaction between PON1 genotype and pesticide exposure on cardiovascular risk factors has not been investigated. We explored if the PON1 Q192R genotype affects cardiovascular risk factors in school-age children prenatally exposed to pesticides.

Abstract

Background: Prenatal environmental factors might influence the risk of developing cardiovascular disease later in life. The HDL-associated enzyme paraoxonase 1 (PON1) has anti-oxidative functions that may protect against atherosclerosis. 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 [4,5]. A common polymorphism, PON1 Q192R, affects both the catalytic activity and the anti-atherogenic properties, but a potential interaction between PON1 genotype and pesticide exposure on cardiovascular risk factors has not been investigated. We explored if the PON1 Q192R genotype affects cardiovascular risk factors in school-age children prenatally exposed to pesticides.

Methods: Pregnant greenhouse-workers were categorized as high, medium, or not exposed to pesticides. Their children underwent a standardized examination at age 6-to-11 years, where blood pressure, skin folds, and other anthropometric parameters were measured. PON1-genotype was determined for 141 children (88 pesticide exposed and 53 unexposed). Serum was analyzed for insulin-like growth factor I (IGF-I), insulin-like growth factor binding protein 3 (IGFBP3), insulin and leptin. Body fat percentage was calculated from skin fold thicknesses. BMI results were converted to age and sex specific Z-scores.

Results: Prenatally pesticide exposed children carrying the PON1 192R-allele had higher abdominal circumference, body fat content, BMI Z-scores, blood pressure, and serum concentrations of leptin and IGF-I at school age than unexposed children. The effects were related to the prenatal exposure level. For children with the PON1 192QQ genotype, none of the variables was affected by prenatal pesticide exposure.

Conclusion: Our results indicate a gene-environment interaction between prenatal pesticide exposure and the PON1 gene. Only exposed children with the R-allele developed adverse cardiovascular risk profiles thought to be associated with the R-allele.
the potential interaction between this genotype and pesticide exposures on cardiovascular risk factors has not been investigated.

We recently reported lower birth weight followed by an increased body fat accumulation from birth to school age in children whose mothers were exposed occupationally to modern non-persistent pesticides in early pregnancy [12]. In this cohort of children, we have determined the PON1 Q192R polymorphism and prenatal pesticide exposure on risk markers for development of CVD later in life.

Methods

Study Population and Design

This study is a part of an ongoing prospective study of the effects of pesticide exposure in early pregnancy on the growth and development in the children. From 1996 to 2000 pregnant women working in greenhouses and referred to the Department of Occupational Health at Odense University Hospital in Denmark for advice regarding their working conditions during pregnancy, were recruited consecutively [13]. Their children were examined for the first time at three months of age [13] and followed up at school age [12].

At enrolment (gestational weeks 4–10), detailed information about working conditions, pesticide use and exposure was obtained from maternal interviews and supplemented by telephone contact to the employers. For all women, re-entry activities (such as moving or packing pot plants or nipping cuttings) constituted their main work functions. Approximately 20% of the women reported to have been directly involved in applying pesticides, mainly by irrigating fungicides or growth retardants. Only few (6%) of the women had applied insecticides.

The total pesticide exposure level was categorized independently (with agreement in all cases)by two toxicologists with special expertise in working conditions in greenhouse horticultures into three groups: none/low (controls), medium, and high as previously described [12,14]. The exposure classification was performed before the first examination of children. Approximately 200 different pesticide formulations, representing 124 different active pesticide ingredients, were used in the working areas. Some were used only in few greenhouses or during restricted time periods, whereas, others were used more often. Three organophosphates: dichlorvos, dimethoate, and chlorpyriphos, were among the 20 most frequently used pesticides, and twelve other organophosphates were used less frequently. For most (91%) of the women rated as pesticide exposed, organophosphates had been used in the working area, but 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). Hence, the re-entry exposure was regarded higher for fungicides and growth regulators than for insecticides. 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, pesticide exposure mainly occurred early in pregnancy.

A total of 168 children were categorized as prenatally pesticide exposed, and 35 children were categorized as unexposed. Of these children 133 (65.5%) accepted an invitation to participate in a follow-up study when the children were between 6 and 11 years old. In this group 112 (59 boys and 53 girls) were prenatally pesticide exposed and 21 (14 boys and 7 girls) were unexposed. To supplement the unexposed group, the participating families were asked to invite children from family, friends and neighbours to participate. Only children between 6 and 11 years whose mother had not been occupationally exposed to pesticides during pregnancy could participate. Through this method, 44 children were recruited [12].

Ethics

The study was conducted according to the Helsinki II Declaration with written informed consent by all mothers and was approved by The Regional Scientific Ethical Committee for Southern Denmark and the Danish Data Protection Agency.

Questionnaire

All families completed a questionnaire prior to the examination of the child with information on medical history, education, occupation, living conditions, life style and diet. An additional short questionnaire regarding smoking, alcohol intake, medical use, disease and occupation during pregnancy was answered by the ‘new’ families for whom we did not have this information from the first examination. Information on gestational age at birth, birth weight and length was obtained from obstetric records.

Social class of the family was coded based on the parents education and occupation according to the criteria of the Danish National Institute of Social Research [15,16], which is almost identical to the UK Registrar General’s classification of five social classes ranked 1 (high) to 5 (low). The social class of the higher ranked parent living with the child was used.

Clinical Examination

The children underwent a standardized physical examination. Body weight [kg] was measured on a digital weight scale with a precision of 0.1 kg (TBF-300, Tanita Europe, UK). Standing height (cm) was measured to the nearest 0.1 cm using a transportable stadiometer (Chasmors LTD., London, UK). Thickness of skin folds (mm) at triceps, subscapular, biceps and flank suprailliac were measured by a caliper (John Bull, British indicators LTD, UK) with a precision of 0.1 mm after allowing the jaws to close on the fat fold for two seconds as described by Rodriguez et al [17]. Abdominal circumference was measured at the level of the umbilicus in a horizontal line with a tape measure to the nearest mm. All anthropometrical measurements were measured in triplicate and means were used for analysis.

Systolic and diastolic blood pressure was measured three times with an automated non-invasive blood pressure monitor (Colin Press-Mate, Colin® Corporation, Japan) and means were used for analysis.

Heart rate was measured on a Heart Rate Monitor (Polar s810) connected to a computer. After the child had been lying in a relaxed, supine position for at least 5 minutes, 300 R-R intervals were measured and 100 consecutive R-R intervals with the lowest standard deviation (SD) were extracted for calculation of the coefficient of variation for heart rate (CVRR). CVRR is the ratio in (percent) of the SD of the R-R intervals to the mean R-R interval (SD/mean R-R interval). Due to technical problems with the Heart Rate Monitor during four of the examination days, heart rate was obtained only for 128 of the 177 children.

The same paediatrician (CWV) performed all clinical examinations blinded to information about maternal pesticide exposure during pregnancy.

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

Laboratory Analysis

Venous non-fasting blood samples were obtained (between midmorning and late afternoon) from 145 of the children. The
samples were collected in EDTA coated and uncoated vials (Venoject). After centrifugation at 2000 g for 10 min at 20°C, buffy coat for genotyping was separated from the EDTA-treated samples. Buffy coat and serum from the uncoated vials were stored at −80°C until analysis.

**Metabolic Biomarkers**

Insulin (proinsulin and insulin) and leptin concentrations in serum were analyzed using commercial hormone kits from RayBio (RayBio Human Insulin Elisa Kit or Human Leptin Elisa kit, cat. no. ELH-insulin-001 human and ELH-leptin-001, AH Diagnostics, Aarhus, Denmark). The assays were performed with immobilized specific antibody-coated 96-well plates. The amounts of both insulin and leptin were quantified using standard curves for each antigen evaluated in the same analysis (for details see the manufacturers instructions). Limits of detection were 6 pg/ml for leptin and 160 pg/ml for insulin and interassay variation was less than 10% and 12% for leptin and insulin, respectively.

Insulin-like growth factor 1 (IGF-I) and insulin-like growth factor binding protein 3 (IGFBP3) were measured in serum with solid-phase enzyme-labelled chemiluminescent immunometric assays (Immulite 2000; Diagnostic Products Corporation, Los Angeles, CA, USA) using World Health Organization (WHO) NIBSC IRR 87/518 and 93/560 standards, respectively. The limits of detection were 25 ng/ml and 500 ng/ml, with intra- and interassay variation less than 5% and 7%, respectively.

**Genotyping of PON1**

DNA was isolated from buffy coats and C-108T (rs705379), M55L (rs854560) and Q192R (rs662) polymorphisms of the PON1 gene were determined by the Taqman-based allele discrimination using the ABI Prism 7700 Sequence Detection System, as previously described [18]. *PON1* genotype was performed successfully for 141 of the children.

**PON1 Activity**

The serum activity of PON1 was determined with paraoxon as substrate as previously described [19,20]. Briefly, serum was added to Tris buffer (100 mmol/L, pH 8.0) containing 2 mmol/L CaCl₂ and 5.5 mmol/L paraoxon (O,O-diethyl-O-p-nitrophenoxyphosphate, Sigma Chemical Co). The rate of p-nitrophenol generation was determined at 405 nm, 25°C, using a continuously recording spectrophotometer (Perkin Elmer, Lambda 11).

**Data Analysis**

Since only 11 children had the RR genotype, those with the QR and RR type were combined in the data analysis. The serum activity of PON1 was determined with paraoxon as substrate as previously described [19,20]. Briefly, serum was added to Tris buffer (100 mmol/L, pH 8.0) containing 2 mmol/L CaCl₂ and 5.5 mmol/L paraoxon (O,O-diethyl-O-p-nitrophenoxyphosphate, Sigma Chemical Co). The rate of p-nitrophenol generation was determined at 405 nm, 25°C, using a continuously recording spectrophotometer (Perkin Elmer, Lambda 11).

**Results**

This paper present the results for 141 children for whom *PON1* genotypes were determined. The genotype frequencies for *PON1* Q192R were 56.7% QQ, 35.5% QR, and 7.8% RR corresponding to an R-allele frequency of 25.5%. The distribution approximated the Hardy-Weinberg equilibrium. QQ homozygous children had significantly lower paraoxonase 1 activity than the R-carriers (Table 1).

None of the other characteristics differed significantly between children with the QQ genotype and the QR/RR genotype. The *PON1* Q192R genotype explained 66% of the variance in the paraoxonase activity. No significant
correlations between paraoxonase activity and any outcome variables were seen (data not shown).

Significant interactions between PON1 Q192R genotype and prenatal pesticide exposure were seen for all outcomes, except systolic blood pressure, CVRR, and non-fasting serum concentrations of insulin, both before and after adjusting for covariates (Table 2). This finding indicates a higher susceptibility towards prenatal pesticide exposure in individuals with the R-allele. In genotype stratified analysis, an exposure-related increase in abdominal circumference, skin fold thickness, body-fat percentage (Table 3), BMI Z-score, BMI Z-score difference from birth to school age (Table 4) and in systolic and diastolic blood pressure (Table 5) was seen in children with the R-allele. CVRR tended to be lower in exposed than in the unexposed R-carriers but the difference was not statistically significant, possibly due to a large variation in the CVRR data. Exclusion of three children with CVRR above 20% and one child with CVRR below 2% did not materially change the results. For QQ-homozygote children, none of the outcome variables was significantly affected by prenatal pesticide exposure.

The mean birth weight was significantly lower for exposed than unexposed children (3521 g versus 3677 g, p = 0.04). This association was seen for both QQ homozygotes and R-carriers although it did not reach significance for the genotypes separately (data not shown). Among the R-carriers, 53.8% of the exposed children had an increase in BMI Z-score of more than 0.67 compared to 13.6% among the unexposed (p = 0.003). For the QQ-homozygotes, the proportion of children with BMI Z-score above 0.67 was similar for exposed and unexposed, 32.7% and 29.0%, respectively (p = 0.81). The low percentage for the group of unexposed R-carriers was due to negative BMI Z-scores at school age and negative difference in BMI Z-scores from birth to school age. Hence, this group of children was leaner than the reference population and also the unexposed QQ-homozygotes. The same pattern was seen for abdominal circumference, sum of four skin folds, body fat percentage, and serum concentration of IGF-I (Tables 2, 3, 4, 5, 6). Systolic and diastolic blood pressure, CVRR, and serum concentration of leptin did not differ between unexposed R-carriers and QQ-homozygotes (Table 5 and 6). In R-carriers, non-fasting serum concentrations of leptin and IGF-I were increased after prenatal pesticide exposure in an exposure

### Table 1. Characteristics of 141 children with genotype PON1 192QQ or QR/RR examined at age 6–11 years.

| Outcome a | PON1 192QQ | PON1 192QR/RR | p-value |
|-----------|------------|--------------|---------|
| No of boys/girls (% boys) | 47/33 (58.8) | 35/26 (57.4) | 1.00 |
| Prenatal pesticide exposure [N (%)] | 0.87 |
| No exposure | 31 (38.8) | 22 (36.1) |
| Medium exposure | 28 (35.0) | 24 (39.3) |
| High exposure | 21 (26.3) | 15 (24.6) |
| New recruited controls [N (%)] | 21 (26.3) | 16 (26.2) | 1.00 |
| Age (years) | 8.7 (6.7; 10.6) | 8.3 (6.8; 10.6) | 0.26 |
| Height (cm) | 134.2 (121.2; 145.5) | 132.9 (116.1; 146.2) | 0.45 |
| Weight (kg) | 29.7 (22.9; 39.5) | 28.4 (19.9; 43.4) | 0.50 |
| Parental social class [N (%)] | 0.29 |
| 1-3 | 28 (35.0) | 14 (23.0) |
| 4 | 37 (46.3) | 33 (47.1) |
| 5 | 15 (18.8) | 14 (23) |
| Birth weight (g) | 3520 (2590; 4677) | 3735 (2295; 4455) | 0.25 |
| Gestational age (days) | 283 (255; 297) | 282 (261; 296) | 0.94 |
| Maternal smoking in pregnancy [no. (%)] | 19 (23.8) | 13 (21.3) | 0.84 |
| Maternal alcohol consumption in pregnancy [no. (%)] | 32 (40.0) | 18 (30.5) | 0.29 |
| Mother of non-Danish origin | 4 (5.0) | 2 (3.3) | 0.70 |
| Paraoxonase activity (nmol/min/ml) | 29.6 (18.5; 29.6) | 58.5 (42.4; 72.1) | <0.001 |
| PON1 55 [N (%)] | <0.001 |
| LL | 22 (27.5) | 31 (51) |
| ML | 38 (47.5) | 30 (49) |
| MM | 20 (25.0) | 0 |
| PON1 108 [N (%)] | 0.15 |
| CC | 19 (23.8) | 21 (34.4) |
| CT | 46 (57.6) | 25 (41.0) |
| TT | 15 (18.8) | 15 (24.6) |

For continuous variables, data represent median (5;95 percentiles).

* Differences between groups were tested with Mann-Whitney U-Test (continuous data) or Fishers Exact Test (categorical data with two categories) or Likelihood Ratio (categorical data with more than two categories).

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### Table 2. Crude and adjusted estimated effects (β, 95%CI) of prenatal pesticide exposure, and PON1 Q192R genotype on anthropometric outcomes, blood pressure, and metabolic biomarkers at school age.

|                                                    | Crude model | Adjusted model |
|----------------------------------------------------|-------------|----------------|
|                                                    | β (95%CI)   | p-value    | β (95%CI)   | p-value    |
| **Abdominal circumference**<sup>a</sup> (cm)        |             |             |             |             |
| PON1 192QR/RR (unexposed)                          | −6.30 (−11.32; −1.00) | 0.02 | −6.22 (−11.01; −1.18) | 0.02 |
| Pesticide exposure (PON1 192QQ)                    | −2.00 (−6.34; 2.54) | 0.38 | −2.26 (−6.40; 2.06) | 0.30 |
| Pesticide exposure (PON1 192QR/RR) (interaction)   | 10.09 (2.71; 18.01) | 0.01 | 10.35 (3.34; 17.85) | 0.004 |
| **Sum of four skin folds**<sup>a</sup> (mm)         |             |             |             |             |
| PON1 192QR/RR (unexposed)                          | −20.12 (−33.4; −2.87) | 0.03 | −21.81 (−35.24; −5.61) | 0.01 |
| Pesticide exposure (PON1 192QQ)                    | −5.88 (−19.88; 10.55) | 0.46 | −7.68 (−20.97; 7.85) | 0.31 |
| Pesticide exposure (PON1 192QR/RR) (interaction)   | 49.01 (16.42; 70.71) | 0.002 | 52.94 (20.80; 93.64) | 0.001 |
| **Body fat percentages**<sup>a</sup> (%)           |             |             |             |             |
| PON1 192QR/RR (unexposed)                          | −15.79 (−27.72; −1.90) | 0.03 | −16.44 (−28.05; −2.95) | 0.02 |
| Pesticide exposure (PON1 192QQ)                    | −4.86 (−16.10; 7.88) | 0.44 | −5.82 (−16.76; 6.56) | 0.34 |
| Pesticide exposure (PON1 192QR/RR) (interaction)   | 35.47 (11.73; 64.62) | 0.002 | 37.00 (13.58; 65.24) | 0.001 |
| **BMI Z-score at school age**<sup>b</sup>          |             |             |             |             |
| PON1 192QR/RR (unexposed)                          | −0.70 (−1.36; −0.04) | 0.04 | −0.78 (−1.43; −0.13) | 0.02 |
| Pesticide exposure (PON1 192QQ)                    | −0.15 (−0.69; 0.39) | 0.59 | −0.26 (−0.80; 0.28) | 0.34 |
| Pesticide exposure (PON1 192QR/RR) (interaction)   | 1.30 (0.47; 2.13) | 0.002 | 1.36 (0.54; 2.17) | 0.001 |
| **BMI Z-score between school age and birth**<sup>c</sup> |             |             |             |             |
| PON1 192QR/RR (unexposed)                          | −0.61 (−1.45; 0.23) | 0.16 | −0.53 (−1.30; 0.24) | 0.18 |
| Pesticide exposure (PON1 192QQ)                    | 0.13 (−0.56; 0.83) | 0.71 | −0.07 (−0.70; 0.57) | 0.83 |
| Pesticide exposure (PON1 192QR/RR) (interaction)   | 1.43 (0.36; 2.49) | 0.01 | 1.32 (0.35; 2.28) | 0.01 |
| **Systolic blood pressure**<sup>d</sup> (mmHg)      |             |             |             |             |
| PON1 192QR/RR (unexposed)                          | −0.61 (−4.75; 3.54) | 0.77 | −0.30 (−4.53; 3.94) | 0.89 |
| Pesticide exposure (PON1 192QQ)                    | −0.67 (−4.09; 2.74) | 0.70 | −0.57 (−3.99; 2.86) | 0.74 |
| Pesticide exposure (PON1 192QR/RR) (interaction)   | 3.28 (−1.97; 8.53) | 0.22 | 3.19 (−2.26; 8.64) | 0.25 |
| **Diastolic blood pressure**<sup>d</sup> (mmHg)     |             |             |             |             |
| PON1 192QR/RR (unexposed)                          | −0.88 (−4.29; 2.53) | 0.61 | −0.50 (−4.00; 3.00) | 0.78 |
| Pesticide exposure (PON1 192QQ)                    | −1.45 (−4.26; 1.36) | 0.31 | −1.30 (−4.13; 1.53) | 0.37 |
| Pesticide exposure (PON1 192QR/RR) (interaction)   | 5.35 (1.03; 9.66) | 0.02 | 4.89 (0.38; 9.40) | 0.03 |
| **CVn**                                            |             |             |             |             |
| PON1 192QR/RR (unexposed)                          | −8.7 (−35.9; 30.0) | 0.61 | −11.2 (−37.8; 26.7) | 0.51 |
| Pesticide exposure (PON1 192QQ)                    | 6.1 (−20.7; 42.1) | 0.69 | 7.2 (−19.8; 43.4) | 0.63 |
| Pesticide exposure (PON1 192QR/RR) (interaction)   | −23.4 (−49.7; 16.4) | 0.21 | −22.4 (−49.0; 18.0) | 0.23 |
| **Leptin**<sup>e</sup> (ng/ml)                     |             |             |             |             |
| PON1 192QR/RR (unexposed)                          | −34.8 (−61.2; 9.3) | 0.10 | −33.9 (−60.4; 10.2) | 0.11 |
| Pesticide exposure (PON1 192QQ)                    | 28.2 (−17.0; 98.0) | 0.26 | 31.3 (−14.5; 101.6) | 0.21 |
| Pesticide exposure (PON1 192QR/RR) (interaction)   | 135.2 (20.8; 357.8) | 0.01 | 141.7 (25.2; 366.7) | 0.01 |
| **Insulin**<sup>f</sup> (ng/ml)                    |             |             |             |             |
| PON1 192QR/RR (unexposed)                          | 14.0 (−31.7; 90.4) | 0.61 | 10.7 (−33.9; 85.4) | 0.70 |
| Pesticide exposure (PON1 192QQ)                    | 31.6 (−14.7; 103.0) | 0.21 | 30.6 (−15.4; 101.8) | 0.23 |
| Pesticide exposure (PON1 192QR/RR) (interaction)   | 11.7 (−41.8; 114.6) | 0.74 | 15.4 (−40.1; 122.4) | 0.67 |
| **IGF-1**<sup>f</sup> (ng/ml)                      |             |             |             |             |
| PON1 192QR/RR (unexposed)                          | −21.3 (−36.2; −2.9) | 0.03 | −20.4 (−34.3; −3.5) | 0.02 |
| Pesticide exposure (PON1 192QQ)                    | −14.8 (−28.3; 1.3) | 0.07 | −13.0 (−25.7; 1.8) | 0.08 |
| Pesticide exposure (PON1 192QR/RR) (interaction)   | 48.9 (14.2; 94.2) | 0.004 | 50.1 (17.9; 91.1) | 0.001 |
| **IGFBP3**<sup>f</sup> (ng/ml)                     |             |             |             |             |
| PON1 192QR/RR (unexposed)                          | −273.9 (−636.2; 88.5) | 0.14 | −253.6 (−605.1; 98.0) | 0.16 |

Prenatal Pesticide Exposure, PON1, and CVD Risk
dependent manner (Table 6). After adjusting for BMI, the effect on leptin, but not IGF-I, remained significant (data not shown). For insulin and IGFBP-3, the results were less consistent. Within the group of children with the QQ genotype, those with the 55MM genotype had higher birth weight than those with the 55LL or 55 ML type (data not shown) but none of the outcome variables measured at school age differed between the \textit{PON1} L55M genotypes. Including the \textit{PON1} L55M genotypes in the regression analysis (modelled as 0 (MM), 1 (ML), and 2 (LL)) did not change the results.

### Discussion

Despite the small sample size, our study found indications of a gene-environment interaction between prenatal pesticide exposure and the \textit{PON1} Q192R genotype that might affect the risk of obesity and related diseases later in life. Prenatally pesticide-exposed children carrying the R-allele had higher abdominal circumference, body fat content, BMI Z-scores, systolic and diastolic blood pressure, and serum concentrations of leptin and IGF-I at school age than did unexposed children. For QQ-homozygous children, none of these variables were significantly affected by prenatal pesticide exposure. The results indicate that R-carriers may be especially susceptible towards developmental disturbances after prenatal pesticide exposure.

An important strength of this study is the longitudinal design that minimizes exposure misclassification and bias. The classification of the mothers as high, medium or not exposed was done independently by two toxicologists with special expertise in working conditions in greenhouse horticultures at enrolment early in pregnancy [13,14] and hence completely independent of subsequent examination outcomes for the children. The unexposed group was extended at the follow-up examination to provide better comparison data. None of the mothers of these children had worked in greenhouses or other occupations where pesticides were used during pregnancy. They were distributed evenly between the group of children with the R-allele and the other genotypes.

### Table 2. Cont.

|                                      | Crude model | Adjusted model |
|--------------------------------------|-------------|----------------|
|                                      | β (95%CI)   | p-value        | β (95%CI)   | p-value        |
| Pesticide exposure (\textit{PON1} 192QQ) |             | 0.61           |             | 0.72           |
|                                      | 72.2 (42.1; 102.2) | 0.05           | 72.3 (42.2; 102.3) | 0.04           |

β expresses the mean differences for BMI Z-score at school age, ΔBMI Z-score between school age and birth, systolic and diastolic blood pressure, and IGFBP3 and the relative difference (in percent) for ln-transformed outcomes (abdominal circumference, sum of four skin folds, body fat percentages, coefficient of variation for heart rate (CV\textit{RR}), leptin, insulin, and IGF-II).

Adjusted model included gender, age at examination, social class, and maternal smoking in pregnancy; social class, and maternal smoking in pregnancy; gestational age, social class, and maternal smoking in pregnancy; gender, age at examination, maternal smoking in pregnancy, and BMI; gender, age at examination, and maternal smoking in pregnancy; gender and age at examination.

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# Table 3. Adjusted values* for anthropometric outcomes at school age in children in relation to prenatal pesticide exposure and \textit{PON1} Q192R genotype.

|                                      | All children | \textit{PON1} 192QQ | \textit{PON1} 192QR/RR |
|--------------------------------------|--------------|---------------------|-----------------------|
| Abdominal circumference (cm)         |              |                     |                       |
| No exposure                          | 62.3 (60.6; 63.9) | 63.2 (61.0; 65.4) | 59.2 (56.8; 61.7) |
| Medium exposure                      | 63.2 (61.6; 64.8) | 61.0 (58.8; 63.3) | 62.4 (60.0; 64.8) |
| High exposure                        | 63.8 (61.9; 65.8) | 62.7 (60.1; 65.4) | 65.9 (62.7; 69.3) |
| Sum of four skin folds (mm)          |              |                     |                       |
| No exposure                          | 40 (36; 44)  | 43 (38; 49)         | 34 (30; 39)          |
| Medium exposure                      | 44 (40; 48)  | 36 (32; 42)         | 44 (39; 50)          |
| High exposure                        | 46 (41; 52)  | 44 (38; 51)         | 52 (44; 62)          |
| Body fat percentages (%)             |              |                     |                       |
| No exposure                          | 18.1 (16.8; 19.5) | 19.1 (17.2; 21.1) | 16.0 (14.4; 17.9) |
| Medium exposure                      | 19.1 (17.8; 20.6) | 16.8 (15.1; 18.7) | 19.5 (17.5; 21.6) |
| High exposure                        | 20.4 (18.7; 22.3) | 19.6 (17.4; 22.2) | 22.3 (19.6; 25.5) |

*p < 0.05
**p ≤ 0.01
***p ≤ 0.001 compared to unexposed.

Results from linear regression model adjusted for gender, age at examination, social class, and maternal smoking in pregnancy. The outcome variables were ln-transformed.

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and the QQ homozygotes, and, therefore, did not affect the differences observed in response to prenatal pesticide exposure between the two genotypes. In addition, all examinations of the children were done blinded to the exposure information and by the same paediatrician using standardized procedures. Genotyping of PON1 and analyses of serum samples were performed blinded to both exposure information and examination outcomes.

The small number of exposed and unexposed children within each of the two PON1 genotypes is a limitation of the study, because it decreases the power of the study and diminishes the possibility of detecting exposure related effects. Nonetheless, if detectable in a small study like ours, the associations may be important. An additional limitation is that we conducted many statistical analyses and chose not to adjust for multiple compar-

| Table 4. Multiple regression analysis of prenatal pesticide exposure, maternal smoking and social class as predictors for BMI Z-scores at school age, and difference in BMI Z-scores between birth and school age in children with the PON1 192 QQ or QR/RR genotype. |
|---------------------------------|---------------------------------|---------------------------------|
|                                | PON1 192QQ                      | PON1 192QR/RR                   |
|                                | β (95% CI)                      | p-value                         | β (95% CI)                      | p-value                         |
| **BMI Z-score at school age**  |                                 |                                 |                                 |                                 |
| Social class 5                 | 0.49 (−0.24; 1.22)              | 0.19                            | 0.07 (−0.69; 0.82)              | 0.86                            |
| Social class 1–3               | −0.43 (−1.01; 0.16)             | 0.15                            | −0.19 (−0.96; 0.59)             | 0.64                            |
| Maternal smoking in pregnancy | 0.10 (−0.54; 0.74)              | 0.77                            | 0.77 (0.02; 1.52)               | 0.04                            |
| Maternal pesticide exposure medium level | −0.45 (−1.06; 0.15) | 0.14                            | 0.79 (0.09; 1.50)               | 0.03                            |
| Maternal pesticide exposure high level | −0.07 (−0.73; 0.58)     | 0.82                            | 1.57 (0.76; 2.37)               | <0.001                          |
| **BMI Z-score between school age and birth** |
| Gestational age (days)         | −0.04 (−0.06; −0.01)            | 0.003                            | −0.07 (−0.10; −0.03)            | <0.001                          |
| Social class 5                 | 0.77 (−0.12; 1.66)              | 0.09                            | 0.21 (−0.68; 1.09)              | 0.64                            |
| Social class 1–3               | −0.77 (−1.50; −0.04)            | 0.04                            | −0.29 (−1.19; 0.61)             | 0.52                            |
| Maternal smoking in pregnancy | 0.33 (−0.44; 1.11)              | 0.39                            | 0.82 (−0.07; 1.70)              | 0.07                            |
| Maternal pesticide exposure medium level | −0.35 (−1.08; 0.39)     | 0.35                            | 1.13 (0.28; 1.99)               | 0.01                            |
| Maternal pesticide exposure high level | 0.15 (−0.66; 0.97)     | 0.71                            | 1.29 (0.36; 2.21)               | 0.007                            |

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| Table 5. Adjusted valuesa for systolic and diastolic blood pressure and coefficient of variation for heart rate (CVRR) at school age in relation to prenatal pesticide exposure and PON1 Q192R genotype. |
|---------------------------------|---------------------------------|---------------------------------|
|                                | Adjusted mean/geometric mean (95% CI) |                                |
|                                | All children                     | PON1 192QQ                      | PON1 192QR/RR                   |
| **Systolic blood pressure (mmHg)** |                                 |                                 |                                 |
| No exposure                    | 100.6 (98.7; 102.6)              | 100.1 (97.4; 102.7)             | 99.1 (95.6; 102.7)              |
| Medium exposure                | 100.1 (98.2; 102.0)              | 99.4 (96.6; 102.3)              | 99.6 (96.6; 102.5)              |
| High exposure                  | 101.7 (99.4; 104.0)              | 99.7 (96.4; 103.0)              | 108.4 (104.1; 112.7)            |
| **Diastolic blood pressure (mmHg)** |                                 |                                 |                                 |
| No exposure                    | 58.9 (57.2; 60.6)                | 58.2 (55.9; 60.4)               | 57.4 (54.8; 60.0)               |
| Medium exposure                | 59.2 (57.5; 60.8)                | 58.1 (55.7; 60.5)               | 61.1 (58.7; 63.4)               |
| High exposure                  | 57.8 (55.8; 59.7)                | 55.5 (52.8; 58.3)               | 62.0 (58.6; 65.5)               |
| **CVRRb**                      |                                 |                                 |                                 |
| No exposure                    | 7.4 (6.3; 8.7)                   | 8.2 (6.3; 10.7)                 | 7.4 (5.8; 9.5)                  |
| Medium exposure                | 7.4 (6.5; 8.4)                   | 8.8 (7.1; 10.8)                 | 6.1 (4.9; 7.5)                  |
| High exposure                  | 7.5 (6.3; 8.8)                   | 8.7 (6.7; 11.2)                 | 6.1 (4.7; 8.0)                  |

*Results from linear regression models adjusted for gender, age at examination, maternal smoking in pregnancy, and for systolic and diastolic blood pressure also BMI. CVRR was ln-transformed.

*p≤0.05,

**p<0.01 compared to unexposed.

*p<0.001 compared to PON1 192QQ in the same exposure group.

Due to technical problems with the Heart Rate Monitor, CVRR was only obtained for 98 (14 QQ and 14 QR/RR unexposed, 23 QQ and 19 QR/RR medium exposed, and 16 QQ and 12 QR/RR high exposed) of the 141 children for whom PON1 genotype was determined.

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**Table 6. Adjusted values** for non-fasting serum concentrations of leptin, insulin, IGF1, and IGFBP3 at school age in relation to prenatal pesticide exposure and *PON1* Q192R genotype.

|                    | All children | *PON1* 192QQ | *PON1* 192QR/RR |
|--------------------|--------------|--------------|-----------------|
| **Leptin (ng/ml)** |              |              |                 |
| No exposure        | 2.06 (1.60; 2.66) | 2.48 (1.73; 3.57) | 1.57 (1.11; 2.22) |
| Medium exposure    | 3.67 (2.81; 4.79)** | 2.92 (1.95; 4.37) | 4.41 (3.13; 6.22) *** |
| High exposure      | 4.55 (3.26; 6.36)*** | 3.77 (2.32; 6.13) | 6.47 (4.07; 10.29) *** |
| **Insulin (ng/ml)**|              |              |                 |
| No exposure        | 0.42 (0.33; 0.54) | 0.40 (0.29; 0.54) | 0.47 (0.30; 0.71) |
| Medium exposure    | 0.67 (0.52; 0.86)** | 0.58 (0.42; 0.81) | 0.90 (0.61; 1.33)* |
| High exposure      | 0.46 (0.33; 0.64) | 0.45 (0.30; 0.67) | 0.35 (0.20; 0.60) |
| **IGF-I (ng/ml)**  |              |              |                 |
| No exposure        | 143.9 (131.0; 158.1) | 158.7 (139.8; 180.1) | 125.1 (108.2; 144.6)### |
| Medium exposure    | 139.0 (126.5; 152.7) | 1270 (111.2; 145.1)* | 155.4 (135.2; 178.6)*# |
| High exposure      | 163.1 (145.4; 183.0) | 154.8 (132.8; 180.5) | 173.0 (144.6; 206.9)** |
| **IGFBP3 (ng/ml)** |              |              |                 |
| No exposure        | 3339 (3169; 351) | 3341 (3212; 3670) | 3169 (2897; 3441) |
| Medium exposure    | 3369 (3198; 3540) | 3209 (2971; 3447) | 3586 (3323; 3849) #* |
| High exposure      | 3592 (3384; 3802) | 3615 (3341; 3890) | 3558 (3219; 3897) |

*Results from linear regression models adjusted for gender and age. Leptin, insulin, and IGF-I were ln-transformed.

*p*≤0.05,

**p*≤0.01,

***p*≤0.001 compared to unexposed.

* p*≤0.05,

** p*≤0.01 compared to *PON1* 192QQ unexposed.

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ions, although they may increase the likelihood of spurious associations. However, several of the outcomes are interrelated and the observed associations between prenatal pesticide exposure and effects in the group of children with the R-allele were very robust being significant both before and after adjusting for covariates. In addition, the magnitudes of the effects were related to the exposure level which again supports a causal association. Finally, the distribution of known risk factors such as social class and maternal smoking in pregnancy did not differ between the children with the R-allele and the QQ-homozygotes.

Another possible limitation of the study is that the *PON1* genotypes of the mothers were not determined. The maternal genotype and related ability to metabolize pesticides might be important for protection of the foetus against exposure. However, only 11 children (6 unexposed and 5 exposed) were homozygous for the R-allele, and for the heterozygous children approximately half of the mothers would be assumed to be QQ homozygotes. Therefore, it is unlikely that the observed associations would be explained by the maternal genotype.

In a previous study, women who worked in floricultures in Mexico and had the RR genotype had higher risk of having children with low birth weight than women with the QQ or QR genotype [24]. In another study, maternal organophosphate exposure during pregnancy was associated with reduced head circumference and head size of the children at birth if the mothers had a low *PON1* concentration but no association was seen between neither maternal nor child *PON1* genotype and head circumference or birth weight or birth length [25]. However, at 12 months of age, negative associations between prenatal organophosphate exposure and cognitive development were seen if the mothers had the QR/RR genotype, although later in childhood children of mothers with the QQ-genotype appeared more affected [26]. In a recent study, shorter gestation and smaller head circumference at birth were associated to low infant, but not maternal, *PON1* enzyme activity [27]. This association was independent of maternal organophosphate exposure in pregnancy but for infants with low activity (and *PON1* -108TT and *PON1* 192QQ genotype) an association between maternal organophosphate exposure in pregnancy and shorter gestational age was seen. Although, the R-allele seems to provide better protection than the Q-allele toward toxic effects of certain organophosphates such as chlorpyrifos [8,28], the capacity of *PON1* allozymes to protect LDL from oxidation might be different, and even the reverse, of the paraoxonase activity, with the lowest capacity for the R-allozyme [1,3].

A weak association between the R-allele and increased CVD risk has been confirmed in most case-control studies, and is supported by meta-analyses [2,9,29–33]. Results from studies on associations between *PON1* 192QQ genotype and risk markers for CVD in healthy populations are inconsistent, as some found no association [34,35] or a higher frequency of the R-allele among obese (BMI ≥20 kg/m²) compared to normal-weight pre-menopausal women [36], while increased blood pressure was associated with the R-allele in women above 60 years [30], and in a rural population in Greece [37]. In general, these studies do not include information about environmental exposures, and discrepancies might be due to unidentified gene-environment or gene-gene interactions. In a recent study the combination of low serum HDL concentration and the RR genotype markedly increased the risk of CVD [38]. This interaction is also supported by the results.
from our study where no marked difference in the risk profile between unexposed R-carriers and QQ-homozygotes was seen. In fact, the R-carriers were leaner than the QQ homozygotes at school age. However, the combination of the R-allele and prenatal pesticide exposure had a pronounced effect on the risk profiles.

In our study, pesticide-exposed children with the R-allele had a higher blood pressure and a lower CVRR, although only the former reached statistical significance. Increased blood pressure was also reported for children, whose mothers were occupationally exposed to pesticides during pregnancy in Ecuador [39]. The PON1 genotype was not determined in these studies, but the R-allele frequency is likely to be higher than in our study, since an R-allele frequency of 40 to 60% has been reported for many populations in Latin and South America [24]. Due to maturation of the cardiac autonomic activity, CVRR increases during gestation and early postnatal life followed by a decline [40]. Prenatal exposure to the neurotoxicant methyl mercury caused decreased CVRR in children at 7 and 14 years of age [41].

Children prenatally exposed to pesticides had lower birth weight than unexposed children as previously reported for the children in this study [12], and also reported in several other studies [42–45]. For children with the R-allele, this was followed by accelerated body fat accumulation until school age. Low birth weight followed by catch-up growth and body fat accumulation during childhood is associated with increased risk of obesity, insulin and leptin resistance and CVD later in life [46–48,49]. There is increasing evidence that prenatal and early postnatal environmental factors might influence the risk [49,50]. In addition, prenatal exposure to low doses of some endocrine disrupting chemicals has been demonstrated to cause obesity in rodents [51–53], and epidemiological studies also have linked exposure to some of these chemicals to obesity and metabolic syndrome in humans [54–56]. The mechanisms behind these associations are not fully understood, but might include epigenetic changes that affect gene expression [57,58] and fetal programming of energy balance [59], perhaps including disturbance of the hypothalamic-pituitary-adrenal axis [60]. In rats, low doses of chlorpyriphos during development caused excess weight gain and leptin and insulin dysregulation later in life [61,62] and neonatal parathion exposure disrupted the production of adipokines, including leptin, that regulate appetite by communicating metabolic status between adipose tissues and the brain [63]. Hence, these effects resemble those seen in the exposed children expressing the R-allele.

In the present study, the mothers were exposed early in their pregnancies to a variety of different pesticides including organophosphates and several fungicides with known endocrine disrupting properties [64,65]. All of these substances had been approved by the national regulatory agency in accordance with European Union legislation. Due to the complex exposure setting, it was not possible to identify specific pesticides as responsible for the effects. They may be due to the combined exposure to several pesticides and maybe also to other ingredients added to the pesticide formulations. For most of the exposed mothers, organophosphates had been used occasionally in the working areas, but 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 been investigated.

In conclusion, this study indicates a gene-environment interaction between prenatal pesticide exposure and PON1 gene heterogeneities that affects cardiovascular risk markers already thought to be associated with the R-allele, as such [2]. Individuals with the R-allele in the 192 position seem particularly susceptible, and the combination of this genotype and prenatal pesticide exposure significantly stimulated fat accumulation from birth to school age, caused higher blood pressure and enhanced serum concentration of leptin and IGF-I. These effects are all related to increased risk for development of metabolic syndrome and CVD later in life. The results also illustrate that a susceptible subgroup of a population could be affected, although effects may not be evident in the entire population.

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Author Contributions

Conceived and designed the experiments: HRA CWV KMM TKJ NES PG CD LC CN KM. Contributed reagents/materials/analysis tools: CD CN LC KM. Performed the experiments: HRA CWV CD CN LC KM. Analyzed the data: HRA CWV KMM TKJ NES PG CD LC CN KM. Contributed writing: HRA CWV CD LC CN KM TKJ NES PG.

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