Title

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Recent progress in the genetics and epigenetics of paraoxonase: why it is relevant to children's environmental health

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Abstract

Purpose of review—Children are more susceptible to exposures *in utero* and during early childhood that may result in developmental problems and chronic diseases. Novel discoveries in the field of molecular epidemiology which can help explain susceptibility to exposures and disease will be demonstrated using the multifunctional enzyme paraoxonase (PON1) as an example.

Recent findings—The broad PON1 variability in humans, partly due to differences in genetics and age, can confer differential susceptibility because this enzyme can detoxify organophosphate pesticides and has antioxidant properties. Epigenetics plays a significant role in the mediation of the effects of environmental exposure on human health and is hypothesized to be a major contributing factor to the early-life origins of adult disease. Studies highlighted in this review demonstrate the relationship of *PON1* polymorphisms with microRNA binding in addition to a link between DNA methylation in the transcriptional regulatory region with changes in PON1 enzyme levels. Other important methodologies such as ancestry informative markers and lactonase activity can enhance studies involving PON1.

Summary—This PON1 model demonstrates that integrating genetic and epigenetic factors as well as other novel methodologies can improve our understanding of important susceptibility factors linked to pediatric disease.

Keywords

differential susceptibility; enzymatic activity; SNPs; oxidative stress; obesity

Introduction

Paraoxonase 1 (PON1) is an enzyme involved in oxidant defense by hydrolyzing oxidized lipids [1] and also plays a key role in detoxification of some organophosphate (OP) pesticides [2]. Thus, individuals with low PON1 levels and activities may be more susceptible to OP exposures and oxidative stress, which occurs when there is an excess of damaging reactive oxygen species compared to the body's antioxidant defense. PON1

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Conflicts of interest

None

genetic variants and lower levels have been linked to adverse health outcomes including oxidative stress related conditions like cardiovascular disease and obesity [3, 4, 5, 6, 7]. Therefore, it is of considerable clinical interest to characterize the protective role of endogenous antioxidant enzymes against the development of obesity and metabolic syndrome (MetS) in children. Previous reviews of PON1 research have shown that age and genetics are key factors associated with PON1 variability and thus susceptibility. Here, we highlight novel developments in PON research including epigenetic mechanisms like DNA methylation and non-coding RNAs as well as the effects of genetic admixture, especially important for studies of minority populations, and the relationship of genetic and epigenetic markers with obesity and metabolic disease.

PON Substrate Specific Activities, SNPs, and Status

Human PON1 enzyme is a 43kDa protein composed of 354 amino acids. It is referred to as a multifunctional enzyme because it can metabolize a number of substrates including some oxon forms of organophosphate pesticides (this is where the name paraoxonase originates from) and aryl esters among others. Spectrophotometric methods have been established for the most commonly used substrates for measurement of PON1 molecular phenotype. These include phenyl acetate and paraoxon, which measure arylesterase (AREase) and paraoxonase (POase) activity, respectively. AREase activity is considered an indirect measure of PON1 protein level and has been highly correlated with Western Blot and ELISA experiments [8, 9]. POase activity reflects both quantity and catalytic efficiency of the enzyme.

More recently, several studies have demonstrated the native substrates for PON1 are likely lactones [10] and the researchers are now beginning to incorporate the use of lactonase substrates like dihydrocoumarin [11**] and 5-thiobutyl butyrolactone (TBBL) in PON1 experiments [11**, 12**, 13]. For instance, Ferre et al.[12**] found that TBBLase, but not POase nor PON1 concentrations determined by ELISA, was significantly associated with obesity status in prepubertal and adolescent children (age 9–15 years) as well as several obesity markers like BMI, body fat percentage, and triglyceride levels (Table 1). Obese children with metabolic syndrome (MetS) had even lower levels of TBBLase than obese children who did not have MetS [12**]. As lactones are the endogenous target of PON1, lactonase activity may be an important and relevant marker of PON1 molecular phenotype particularly for studies related to oxidative stress rather than OP pesticide exposure.

The polymorphic *PON1* gene is a member of the *PON* family cluster of genes including *PON1*, *PON2*, and *PON3*, which all reside adjacent to each other on chromosome 7. *PON1* specifically has been mapped along the chromosome 7q21.3-22.1 region [17, 18] and contains nine exons. Over 200 gene variants have been identified in the *PON1* gene, however only a few promoter single nucleotide polymorphisms (SNPs) significantly influence protein levels as measured by arylesterase (AREase) activity [19, 20, 21, 22, 23, 24]. In particular, rs705379 ($PON1_{-108}$), is the strongest predictor of AREase activity. Furthermore, the $PON1_{-108CC}$ genotype is associated with two-fold higher PON1 enzyme levels compared to the $PON1_{-108TT}$ genotype [25, 26]. The SNP at the position 192 (rs662) in the coding region impacts the catalytic efficiency of the enzyme in detoxifying OP

pesticides with the *PON1*$_{192QQ}$ genotype coding for the enzyme with the lowest efficiency [27]. A number of studies have examined the association of numerous *PON1* SNPs with PON1 molecular phenotype but the majority of SNPs do not explain a substantial amount of additional variation of enzyme quantity or activity in comparison to the commonly studied *PON*$_{-108}$ and *PON1*$_{192}$ SNPs [23, 28]. One recent study identified a few rare *PON1* variants and some trans-acting variants located in the *FTO* and *SERPINA12* genes that displayed a modest association with AREase activity independent of common *PON1* SNPs [29]. However, these associations were not as strong as those with previously identified *PON1* SNPs.

Although most studies of PON1 focus on just a small set of the most common PON1 SNPs, one recent study characterized 16 *PON1* genetic variants and their relationship with several different substrate-specific activities including lactonase (using dihydrocoumarin substrate), POase, AREase, and diazooxonase (DZOase) activities in prepubertal children (ages 4–16 years) [11**]. The relationships of these SNPs with the different substrate-specific PON1 activities were highly dependent on their location in the gene. For instance, promoter region SNPs were highly associated with AREase, lactonase, and DZOase while 3′UTR SNPs were more strongly associated with POase and less associated with other substrate-specific activities. Interestingly, Ruperez et al also identified an intronic SNP, rs854566, which was inversely associated with obesity and also significantly associated with all four substrate-specific activities measured. None of the PON1 activities differed between obese and non-obese children, however lactonase activity was correlated with markers involved in lipid metabolism like HDL and ApoA1 [11**].

Several studies have demonstrated that PON1 status, which includes measures of both *PON1*$_{192}$ genotype, which affects catalytic efficiency towards some substrates, and protein levels(AREase assay), may be a more comprehensive descriptor of PON1 molecular phenotype and a more accurate predictor of disease [17, 30, 31].

Other Genetic Effects: Admixture and Ancestry Informative Markers

In genetic association studies of admixed populations, heterogeneity of genetic background can lead to spurious associations if ancestry is related to both a candidate gene and the disease outcome of interest (Figure 1A); this is also referred to as genetic confounding due to population stratification [32]. Structured association methods enable us to adjust for potential genetic confounding. This is done by genotyping a number of Ancestry Informative Markers (AIMs) genetic variants known to vary widely between different ethnic groups and using the known frequencies among reference populations to estimate proportion of ancestry in individuals. The estimated parameters can then be included in statistical models as covariates. Figure 1B and 1C show examples of proportional ancestry distribution estimated by genotyping of over 100 AIMs for two different studies of Latino populations, CHAMACOS [15**] (Mexicans) and GALAII [33]. Ancestral distributions range quite broadly within populations in both studies and between populations for GALAII.

Both functional PON1 genetic variants *PON1*$_{192}$ and *PON1*$_{-108}$, among others vary substantially among ethnic groups. For instance, the frequency of the Q allele for the

PON1$_{192}$ SNP is 0.73 for Caucasians [34], 0.37 for African-Americans [34] and 0.48 for Mexicans [22, 35]. Furthermore, many of the oxidative stress related health outcomes ranging from birth weight to obesity and cardiovascular disease differ in prevalence by ethnic groups, making genetic ancestry an important factor to consider in PON1 studies, especially in admixed populations. We recently examined associations of PON1, obesity, and genetic ancestry in young Mexican-American children [15**]. Although a trend of higher African ancestry with higher BMI Z-scores and odds of obesity was observed, these relationships did not reach statistical significance after adjusting for multiple testing. We also identified a strong increased odds of obesity in children with the *PON1*$_{192QQ}$ genotype at ages 2 and 5 and found similar trends in relation to waist circumference at age 5. After controlling for genetic ancestry, this relationship with *PON1*$_{192}$ genotype remained although beta coefficients changed moderately (9–15%), demonstrating suggestive evidence of genetic confounding by population stratification. Presence of genetic confounding may explain some of the inconsistencies between reported studies as it can introduce bias, yet to our knowledge, few other studies have attempted to adjust for population stratification in *PON1* genetic association studies [11**, 36]

PON1 Epigenetics

PON1 genetics does not completely explain the broad variability of PON1 molecular phenotype and other factors should be considered. There is a growing recognition that epigenetics may play an important role in key biological processes and mechanisms of disease development [37, 38, 39]. Epigenetic mechanisms regulate gene expression without changes in DNA sequence and include DNA methylation, histone modifications, and non-coding RNAs [40, 41, 42]. Here, we focus on DNA methylation and miRNA because in contrast to chromatin modification assays which are mainly qualitative and require large sample volumes, the state of the art methodologies available for these marks are more amenable to human population studies. Furthermore a 2011 review of PON1 research describing potential regulators of PON1 expression highlighted epigenetics as an unexplored field [43].

DNA methylation is the most extensively investigated epigenetic mechanism and refers to the potential of a cytosine (C) base to be methylated at its 5th carbon if followed by a guanine (G) base in the DNA code, called a CpG site. The human genome contains about 30 million CpG sites. CpG sites are distributed throughout several regions of the genes referred to as CpG islands, shores, shelves, and gene bodies. CpG islands are stretches of DNA with a high frequency of CpG dinucleotides that often occur in proximity to gene promoter regions [44]. It was previously believed that the majority of functional changes occurred in CpG islands, but new research has shown that DNA methylation changes along CpG shores (regions within 2kb of islands) and within the gene body may also have functional effects on gene expression [45, 46]. The amount and patterns of DNA methylation are established during the prenatal period and may vary by tissue and cell type [47, 48]. Gain or loss of DNA methylation, referred to as hyper- and hypo- methylation can lead to gene silencing or overexpression, respectively [49].

The *PON1* promoter has one CpG island comprising 19 CpG sites with a second island located near exon 7 (8 CpG sites). There are a total of 287 CpG sites in *PON1* including 66, 48, and 146 CpG sites within shores, shelves, and open sea regions, respectively (Figure 2). Data on PON1 methylation are scarce. Only one study has examined associations between PON1 methylation at several CpG sites with molecular phenotype [50**]. De la Iglesia and colleagues reported an inverse association between methylation levels of several promoter region CpG sites and AREase activity in 47 adults with MetS features in an energy-restricted dietary weight-loss intervention. This relationship was strongest for CpG sites that were closest in proximity to the PON1 transcription start site. Additionally, a parallel decrease in AREase activity was seen with decreases in several obesity-related parameters such as BMI, fat mass, blood pressure, and triglyceride levels. Another small study of 24 adults also reported an association between methylation at two PON1 promoter CpG sites with body weight and waist circumference, providing further evidence that PON1 DNA methylation may influence obesity risk [51].

MicroRNAs (miRNAs) are small (~23 nucleotide) noncoding RNAs that regulate gene expression by pairing with protein-coding mRNAs and directing their posttranscriptional repression. To date, ~2500 miRNAs have been identified in humans however the majority of their target binding sites are not yet known [52]. Putative binding sites within coding sequences can be identified by sequence complementarity to candidate miRNAs [53]. MiRNAs regulate protein expression by cleavage of homologous mRNA or by specific inhibition of translation. It is estimated that 10–30% of human protein-coding genes are targets of miRNA binding [54], and aberrant expression of miRNA has been implicated in numerous diseases [55, 56]. MiRNAs are an excellent epigenetic biomarker to study because: (1) they are ubiquitously expressed in tissues and body fluids including blood, urine, and saliva; (2) since miRNAs are released into the bloodstream from target tissues (i.e. brain, liver) circulating miRNAs may reflect profiles of target tissue [56]; (3) they are highly stable and resistant to RNase activity as well as effects of pH and temperature in stored specimens over time [57].

In silico analyses using miRanda software and considering conservation and good SVR scores have yielded 25 putative miRNA binding sites in the *PON1* 3′ untranslated (UTR) region (Figure 2). One recent study showed that a *PON1* SNP located in a different miRNA binding site (miR-616) was associated both with changes in PON1 expression and increased risk of ischemic stroke and carotid atherosclerosis [58**]. Unlike the other putative miRNAs that may target *PON1*, this is the only miRNA that has been functionally validated and shown by reporter assay to bind *PON1*. Furthermore, Lui and colleagues were able to show that the *PON1* SNP, rs3735590, affected binding affinity of miR-616 to *PON1* and resulted in differences in PON1 expression. These data demonstrate the molecular mechanisms through which the interplay of genetics and epigenetics influence PON1 expression and demonstrate further the clinical significance of PON1 variability. Although the complex interactions between DNA methylation, miRNA, and genetics are not yet well understood, recent data suggest that miRNA gene expression can also be regulated by aberrant DNA methylation of miRNA genes [59, 60, 61, 62].

PON, obesity and metabolic syndrome

Few studies have examined the associations of PON1 substrate-specific activities or genotypes with obesity or MetS in children however this field of research has grown substantially over the past two years (Table 1). AREase activity, a marker of PON1 enzyme quantity, was inversely associated with obesity status [63] and waist circumference [16*] among two studies in children and adolescents. Yet one study of older children in Spain, which measured PON1 concentration by ELISA [12**], and our study in 2 year old Mexican-American children [15**] both found positive associations of PON1 quantity with obesity. Interestingly at age 5, we found that AREase activity was inversely associated with obesity status in $PON1_{192QQ}$ but not $PON1_{192RR}$ children, indicating the associations with obesity can change with age and genotype [15**]. Lactonase (TBBLase) and PON1 specific activity (POase divided by PON1 concentration) were also inversely associated with several obesity parameters (BMI, % body fat) in one study [12**]. The only other study to examine lactonase activity (dihydrocoumarin) did not observe associations of lactonase or other substrate-specific activities with obesity in prepubertal children, but did report a relationship of lactonase activity with lipid profiles [11**].

Two studies, including ours, found higher BMI Z-scores, waist circumference, and odds of obesity with the $PON1_{192Q}$ allele in young children [15**] as well as school age children[14]. However, some studies did not observe the same relationship [11**, 12**]. Furthermore, Ruperez et al.[11**] identified a different intronic *PON1* SNP which was significantly associated with several substrate-specific PON1 activities (DZOase, AREase, and lactonase) as well as obesity. Ruperez et al.[11**] and Ferre et al.[12**] also examined associations with haplotypes, which incorporate combinations of multiple SNPs on a single chromosome, [64]. Nevertheless, based on our study [23] and recent publications that also examined PON1 haplotypes [11**, 12**], there is no apparent advantage in using haplotypes in PON1.

Perspectives

Although findings have been inconsistent, a number of studies have identified potential relationships between *PON1* genetic variants and/or substrate-specific activity with obesity and MetS. Several factors may help to explain differences observed between studies. First, most studies included subjects with large age ranges (more than 2 years) but did not adjust for age in their analyses with substrate-specific activities even though it has been shown that PON1 levels and activities increase with age through at least age 7 [65, 66]. Additionally, it is possible that the relationship of PON1 with obesity may differ by age. Another important difference between studies was the ethnic composition of the populations included, which may be particularly relevant since allele frequencies of many common PON1 SNPs vary so widely between ethnic groups. For future studies, it will be important to adjust for potential confounding by population stratification in PON1 studies related to obesity, particularly in admixed populations. Finally, it should be pointed out that no studies of children's obesity have looked at the relationship of PON1 epigenetics with obesity despite solid preliminary evidence that DNA methylation and miRNAs may affect PON1 variability. Lui and colleagues demonstrated that the complex interactions between genetics and epigenetics are

an important molecular mechanism that affects PON1 expression and important oxidative stress related health outcomes in adults [58**]. Overall, incorporation of novel emerging methodologies and analyses, like epigenetics, lactonase activity, and control for genetic confounding, into PON1 research may help to further characterize molecular mechanisms affecting PON1 variability and susceptibility to OP exposure and oxidative stress related health conditions.

Conclusion

PON1 serves an important model of a susceptibility factor, whose characterization can help us to understand etiology of multiple diseases. Here we highlighted several methodologies that may broaden the scope of PON1 research and help to explain some of the molecular mechanisms linking PON1 to disease. More generally, many of these concepts can be applied to other susceptibility markers involved in metabolism, inflammation, or other important biological pathways. Identifying the factors that modulate their gene expression, accounting for differences in study populations, and trying to assess the complex interplay of different molecular markers (genetics, epigenetics, enzyme levels etc.) may increase our understanding of these susceptibility markers. This is essential for elucidation of the etiology of complex diseases in children and reliable interpretation of data used for personalized medicine.

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Papers of particular interest, published within the annual period of review have been highlighted as:

* of special interest

** of outstanding interest

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Key points

1. The broad variability of PON1 levels and activities in humans can confer differential susceptibility because this multifunctional enzyme can detoxify organophosphate pesticides and has antioxidant properties.
2. Genetics and age are strong determinants of PON1 variability and therefore susceptibility to oxidative stress and OP exposure.
3. Epigenetic modifications like DNA methylation and miRNAs may provide additional insights into mechanisms of PON variability.
4. Future studies of PON1 function should determine lactonase specific activity as a reliable indicator of PON1 activities, in addition to commonly used measurements for PON1 status as well as arylesterase and paraoxonase activity.
5. In order to use PON1 model to predict risk of obesity and other health outcomes, it should integrate enzyme activities, genetic and epigenetic factors, and take into account genetic ancestry as a potential confounding factor.

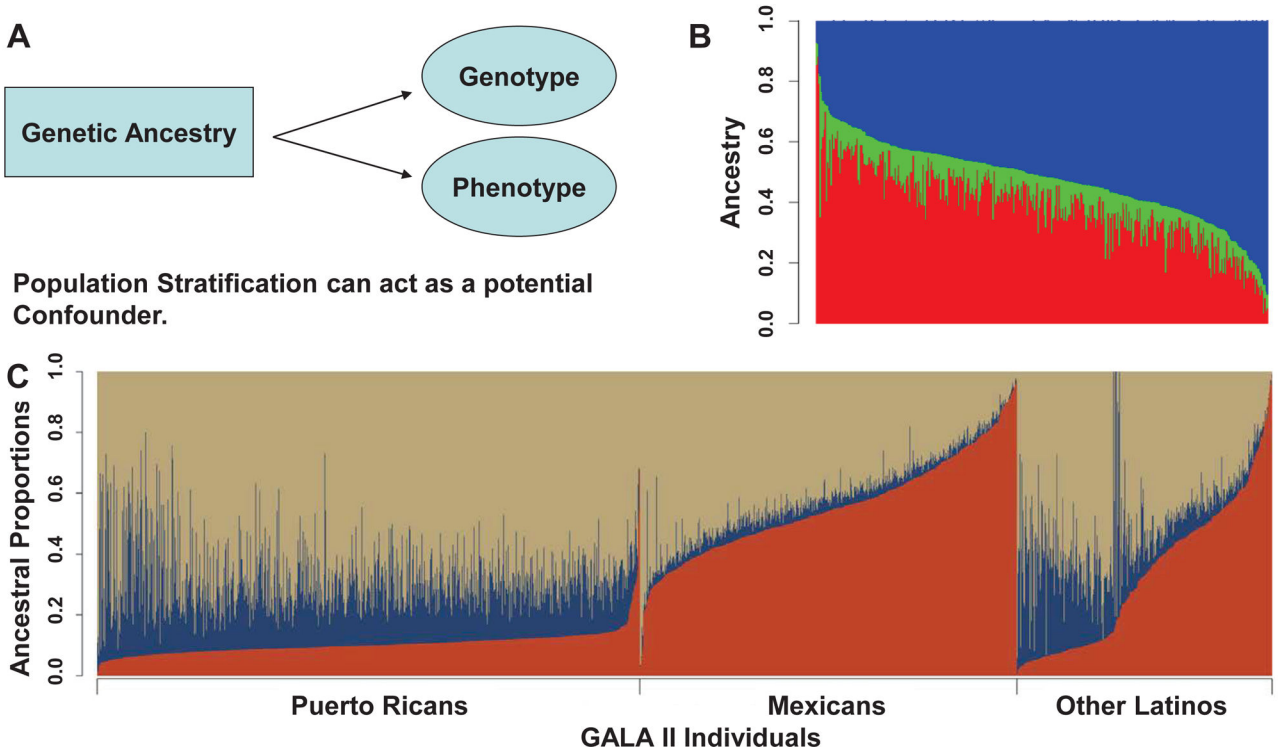

Figure 1.

Figure 1A shows that if differences in ancestry are associated both with genotype and the outcome of interest, genetic ancestry can act as a source of genetic confounding. **Bar plot of genetic ancestry esimates generated by STRUCTURE software.** Estimates were expressed as proportion of European, African and Native American ancestry in B) Mexican-American CHAMACOS children (n=375) and C) GALA II participants of Hispanic origin (; n=6021). Each vertical bar represents the ancestral distribution in 1 subject. For each subject, the proportions of: A) Native American (blue), African (green), and European (red) ancestry and B) Native American (red), African (blue), and European (tan) ancestry are displayed. There is substantial variability in individual ancestry both within and between Hispanic ethnic subgroups. Particularly for studies involving admixed populations, it is important to control for the potential bias introduced by population stratification. This is vital in *PON1* genetic studies because allele frequencies of many common *PON1* SNPs vary between ethnic groups and health outcomes of interest such as obesity and cardiovascular disease also differ by ethnic group. Figure 1B is reproduced with permission from [15**] and Figure 1C is reproduced with permission from [33].

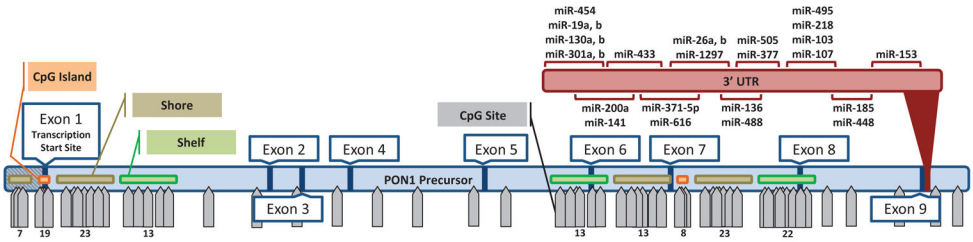

Figure 2. Map of *PON1* CpG sites putative miRNA binding sites

PON1 contains 2 CpG islands, one in the promoter region and one in exon 7. It has 278 CpG sites spread across the CpG islands, shores, shelves, and open seas. *In silico* analyses using the most recent version of miRbase (version 21) has identified 25 putative miRNA binding sites in *PON1*. However, thus far, none of these sites has been functionally validated with *PON1*.

Table 1

Summary of studies related to PON, obesity and metabolic syndrome in children.

Authors	Study population	Age (yrs)	PON1 activities measured	PON associations with obesity and MetS	SNPs
Andersen et al. 2012 [14**]	141 children (88 pesticide exposed and 53 unexposed)	**6–11**	Paraoxonase	NS	rs662 (*PON* $_{192QQ}$) (+O) (+WC)
Huen et al. 2013 [15**]	373	**2 and 5**	Arylesterase	Arylesterase (+O) in 2 yrs old	2 yrs-rs662 (*PON* $_{192QQ}$) (+O) 5 yrs-rs662 (*PON* $_{192QQ}$) (+WC)
Ferre et al. 2013 [12**]	110 obese children and adolescents	**9–15**	Paraoxonase Lactonase	Paraoxonase (-O) Lactonase (-O)(-MetS)	NS
Krzystek-Korpacka et al. 2013 [16*]	156 children and adolescents (47 NW, 27 OW and 82 obese).	**14 ± 2**	Arylesterase	Arylesterase (-WC)	NS
Ruperez et al. 2013 [11**]	189 NW and 179 obese prepubertal children	**4–13**	Paraoxonase Lactonase Arylesterase Diazonase	NS	rs854566 (-O)

Abbreviations used: Obesity (O); Metabolic syndrome (MetS); waist circumference (WC); normal weight (NW); overweight (OW); negative or positive association with obesity and other health outcomes is indicated by (−) or (+) symbols. Studies with no significant relationships found are marked NS.