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Peer-reviewed author version

LUYTEN, Leen; SAENEN, Nelly; JANSSEN, Bram; VRIJENS, Karen; PLUSQUIN, Michelle; ROELS, Harry; Debacq-Chainiaux, Florence & NAWROT, Tim (2018) Air pollution and the fetal origin of disease: A systematic review of the molecular signatures of air pollution exposure in human placenta. In: ENVIRONMENTAL RESEARCH, 166, p. 310-323.

DOI: 10.1016/j.envres.2018.03.025
Handle: http://hdl.handle.net/1942/27589

Air pollution and the fetal origin of disease: A systematic review of the molecular signatures of air pollution exposure in human placenta

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Running title: Placental -omics and air pollution

ABSTRACT

Background Fetal development is a crucial window of susceptibility in which exposure-related alterations can be induced on the molecular level, leading to potential changes in metabolism and development. The placenta serves as a gatekeeper between mother and fetus, and is in contact with environmental stressors throughout pregnancy. This makes the placenta as a temporary organ an informative non-invasive matrix suitable to investigate omics-related aberrations in association with *in utero* exposures such as ambient air pollution.

Objectives To summarize and discuss the current evidence and define the gaps of knowledge concerning human placental -omics markers in association with prenatal exposure to ambient air pollution.

Methods Two investigators independently searched the PubMed, ScienceDirect, and Scopus databases to identify all studies published until January 2017 with an emphasis on epidemiological research on prenatal exposure to ambient air pollution and the effect on placental -omics signatures.

Results From the initial 386 articles, 25 were retained following an *a priori* set inclusion and exclusion criteria. We identified eleven studies on the genome, two on the transcriptome, five on the epigenome, five on the proteome category, one study with both genomic and proteomic topics, and one study with both genomic and transcriptomic topics. Six studies discussed the triple relationship between exposure to air pollution during pregnancy, the associated placental -omics marker(s), and the potential effect on disease development later in life. So far, no metabolomic or exposomic data discussing associations between the placenta and prenatal exposure to air pollution have been published.

Conclusions Integration of placental biomarkers in an environmental epidemiological context enables researchers to address fundamental questions essential in unraveling the fetal origin of disease and helps to better define the pregnancy exposome of air pollution.

Keywords: placenta, air pollution, child development, -omics, Barker hypothesis

ABBREVIATIONS

3-NTp:	3-nitrotyrosine
8-oxodG:	(8-oxo-2'-deoxyguanosine)
AHH:	Aryl hydrocarbon hydroxylase
BC:	Black carbon
BDNF:	Brain-derived neurotrophic factor
CI:	Confidence interval
CYP1A1:	Cytochrome P450 1A1
ECOD:	7-ethoxycoumarin O-deethylase
GST:	Glutathione S-transferase
GSTM1:	Glutathione S-transferase M1
LEP:	Leptin
miRNA:	MicroRNA
MT:	Metallothionein
mtDNA:	Mitochondrial DNA
NAT2:	N-acetyl transferase 2
NO_2:	Nitrogen dioxide
PAH:	Polycylic aromatic hydrocarbon
PECO:	Population, Exposure, Comparator, and Outcome elements
PM:	Particulate matter
$PM_{2.5}$:	Particulate matter with a diameter smaller than 2.5 μm
PM_{10}:	Particulate matter with a diameter smaller than 10 μm
$SO_{2:}$	Sulfur dioxide
SYN1:	Synapsin 1

1. INTRODUCTION

Both genetic and environmental factors contribute to a multitude of complex diseases, while the precise environmental causes and early pathophysiological mechanisms of these diseases remain poorly understood (Ellis *et al.* 2014). The development of diseases can find its origin in every stage of human life. However, the distinct time windows, i.e. pregnancy, infancy, adolescence, adulthood, and old age are characterized by differences in age-specific susceptibilities (Cohen Hubal *et al.* 2008). During the last decade, a major public health concern has focused on the pregnancy period during which the exposure to harmful substances should be avoided to give the newborn the chance to start life as healthy as possible (Sun *et al.* 2016).

Over the entire intrauterine period, the placenta plays a crucial role for growth, development, and survival of the fetus (Burton *et al.* 2016). After the syncytiotrophoblast cells of the blastocyst have invaded the uterine wall, the placenta starts to grow with the formation of chorionic villi, which constitute the fetal side of this temporary organ (Figure 1). One of the first functions of placental cells is to suppress the maternal immune system in such a way that the developing embryo is not rejected (Nugent and Bale 2015). In later stages of pregnancy, the placenta develops a wide spectrum of functions to ensure proper fetal growth. It is endowed with an important transport function mediating the transfer of oxygen, nutritional components, growth factors, and hormones from mother to child, while carbon dioxide and other waste substances are transferred in the opposite direction (Levkovitz *et al.* 2013). This may occur by means of simple diffusion, (energy driven) transporter proteins, and endo- or exocytosis within complex matrices of different cell types, such as trophoblasts, amniotic cells, endothelium lining of the placental blood vessels, decidual cells, Hofbauer cells, and mesenchymal cells (Burton *et al.* 2016).

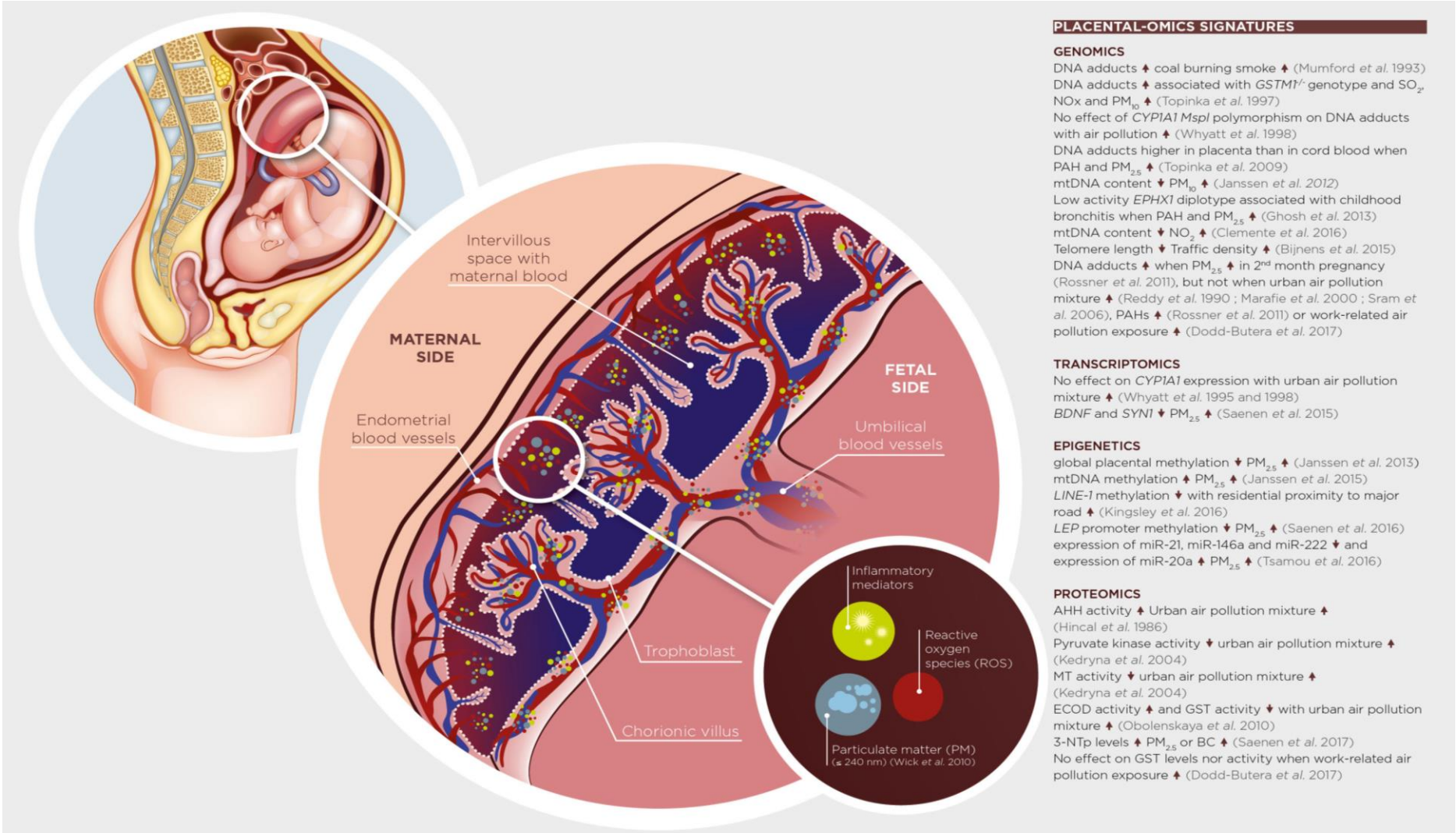

Figure 1: Placental migration of direct (particulate matter) and indirect (reactive oxygen species and inflammatory mediators) potential effectors of exposure to air pollution during pregnancy. The column on the right summarizes the -omics characteristics (genomics, transcriptomics, epigenetics, and proteomics) as described in this systematic review in association with exposure to *in utero* ambient air pollution.

In this way, the placenta comes in contact with, contains and interacts with the substances to which both mother and fetus are exposed to during the timespan of the entire pregnancy. In addition, the placenta itself is an important endocrine organ regulating the production of hormones such as progesterone, human chorionic gonadotrophin (hCG), and human placental lactogen (hPL), to ensure the continuation of pregnancy and to acquire the appropriate maternal responses to optimize the development of the fetus (Burton *et al.* 2016; Nugent and Bale 2015). Furthermore, within the feto-placental unit, a great number of signals are sent from the placenta to the fetus - and *vice versa* - to regulate developmental processes (Dötsch *et al.* 2010). Such signals can also elicit the appropriate reactions to various environmental exposures. Together, all these properties make the placenta an essential organ for the regulation of fetal development. Indeed, placental dysfunction has been linked to for example the occurrence of preeclampsia and adverse birth outcomes such as intrauterine growth restriction (Cha and Kim 2010).

Intrauterine exposure to pollutants can lead to altered metabolic functions that may be detrimental for fetal development. For example, the embryonic brain has a great plasticity and its development depends on, and can be influenced by, various environmental factors (Buss *et al.* 2013). The etiology of diseases in adulthood may have a fetal origin and may be attributed to the effects of adverse environmental exposures *in utero*. This causality concept is known as the Barker hypothesis or the Developmental Origins of Health and Disease (DOHaD). Professor David Barker was the first to recognize this potential link when he became concerned about the association between malnutrition during pregnancy and the development of coronary heart disease in adult life (Barker 1995). Since then, many implications of this hypothesis have been reported (Deng et al. 2016, 2017; Lu et al. 2017). Adverse environmental exposures during pregnancy already identified in this context are active and passive cigarette smoke (Mund *et al.* 2013), and exposure to ambient air pollution [including nitrogen dioxide (NO_2) (Ballester *et al.* 2010), polycyclic aromatic hydrocarbons (PAH) (Jedrychowski *et al.* 2015), and particulate matter (PM) (Rappazzo *et al.* 2014)]. Particles with a diameter smaller than 500 nm are known to pass the placental barrier during the gestational period, while particles with a diameter

123 smaller than 240 nm are even able to reach the fetal bloodstream (Wick *et al.* 2010) (Figure 1), possibly
124 affecting the newborn's metabolism before birth.

125 Various reviews have already described the associations between prenatal ambient air
126 pollution exposure and birth outcomes such as prematurity and birth weight (Lamichhane *et al.* 2015;
127 Shah and Balkhair 2011). However, none of these reviews described the placenta as an intermediate
128 matrix having the potential to express distinct biological (-omics) signatures associated with prenatal
129 exposure to ambient air pollution. Hence, the goal of this systematic review is to provide a structured
130 overview and an evaluation of the current knowledge on the potential of placental tissue as a non-
131 invasive biological matrix for the study of molecular -omics signatures that are associated with *in utero*
132 exposure to ambient air pollution and are probably useful as early-life markers of disease development
133 later in life. With this systematic review we aim to identify signatures in the -omics fields that already
134 have been well addressed and those of which a substantial gap of knowledge still remains in the scope
135 of epidemiological research involving the placenta as a tissue to identify sentinel biological effects of
136 air pollution exposure during pregnancy.

2. MATERIALS AND METHODS

The goal of this systematic review was to provide an answer to the question: "Which -omics biomarkers have been analyzed in human placental tissue used as a non-invasive matrix in epidemiological research in association with prenatal exposure to air pollution in the context of disease development later in life?". The PECO elements that can be deduced from this question were used to determine the selection criteria to search and structure the articles for the synthesis of this review. These PECO elements are:

- "Population": human. In this article we focused on research conducted in an epidemiological context, thus not including research on human cell lines.
- "Exposure": ambient air pollution [including particulate matter with particles smaller than 2.5 µm ($PM_{2.5}$), particulate matter with particles smaller than 10 µm (PM_{10}), ultrafine particles, black carbon (BC), derivatives of nitrogen oxide (NOx), and polycyclic aromatic hydrocarbons (PAHs)]. We defined ambient air pollution as a mixture of indoor and outdoor pollutants, in both solid and gaseous form, and we excluded direct (maternal) or indirect (environmental) exposure to tobacco smoke from this concept.
- "Comparator": in this review were included both studies in which comparisons are made between groups exposed to either a higher or a lower concentration of air pollution, as well as studies with a continuous exposure scale.
- "Outcomes": placental -omics biomarkers and, if discussed, disease development or the development of adverse birth outcomes.

This systematic review was constructed according to existing guidelines on the structure of systematic reviews and maps (Bates *et al.* 2007). An online database search was performed in January 2017, according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (http://www.prisma-statement.org) to identify articles that are dealing with the scope of this review, without any limitation set on the publication date. Two investigators (LJL and NDS) were

appointed to conduct the literature search, because of their expertise on the effects of air pollution in the placenta. These investigators read all papers, extracted, and archived the relevant information independently. The level of consensus between LJL and NDS was determined by performing a Cohen's kappa analysis. Any remaining discrepancies were resolved by consensus. The exploration was conducted on PubMed (http://www.ncbi.nlm.nih.gov/pubmed), Scopus (http://www.scopus.com/), and ScienceDirect (http://www.sciencedirect.com). Only English MeSH-terms were used to form the search strings. First, a search was conducted with the key terms "placenta" and "air pollution". Next, additional searches were performed by replacing these terms with related search queries (for a list of all used queries see Supplemental Tables S1 and S2). Additionally, since we were interested in the link between -omics in the placenta and the development of disease, we replaced the air pollution-related MeSH-terms with the MeSH-terms "fetal origin adult disease", "barker hypothesis", "barker hypothesis fetal" and "barker hypothesis fetal origins" in the identification phase (see Supplemental Table S1 and S2). Only primary research was included in this paper: in case a review article was found in the literature search, the list of references in this review was checked manually to determine if additional articles could be identified that met the inclusion criteria of this systematic review. If a full text could not be obtained, a request was sent via ResearchGate (https://www.researchgate.net/) or via the website of the journal in which the article was published. In search for potential additional information from grey literature, we used a popular search engine (http://www.google.com), and accessed the OpenGrey (http://www.opengrey.eu), and Cochrane Library (http://onlinelibrary.wiley.com/cochranelibrary/) websites. First of all we read the abstract of all papers that were found from the identification procedure and excluded the research articles on animals or human cell lines, since we wanted to put the emphasis solely on epidemiological research. The comparison of differences in placental -omics signatures between different (animal) models is beyond the scope of this systematic review. We also excluded comments on other research articles and the papers not written in English to avoid potential misinterpretation of the results due to incorrect translation. Subsequently, we examined the full text of the remaining articles and excluded

188 those studying exclusively the effect of maternal active and/or passive smoking during pregnancy on
189 placental -omics signatures or fetal health. These articles were excluded because air pollution is a
190 complex mixture that takes into account the effects of various sources, such as traffic- and industry-
191 related pollution, while research on smoking only focusses on the effects of tobacco use. Additionally,
192 research articles that did not consider the measurement of -omics markers in the placenta were not
193 included, because this review specifically focusses on the effects of air pollution exposure during
194 pregnancy on the -omics biomolecular signatures of the placenta. For the remaining articles that were
195 included in this systematic review, the content was examined in detail with a great focus on (i) the
196 placental -omics marker(s) studied and the techniques used to measure them, (ii) the characteristics
197 of prenatal exposure to ambient air pollution in association with the placental -omics marker(s), and
198 (iii) whether the authors mentioned any association with disease development later in life. Finally, a
199 descriptive analysis of these articles was made and a summary of the current knowledge has been
200 provided based on the different -omics fields (genomics, epigenetics, transcriptomics, proteomics and
201 metabolomics). In this way, existing gaps of knowledge in this research field could be established.

3. RESULTS

Using the initial MeSH-terms “placenta” and “air pollution”, 118 articles could be identified (Figure 2).

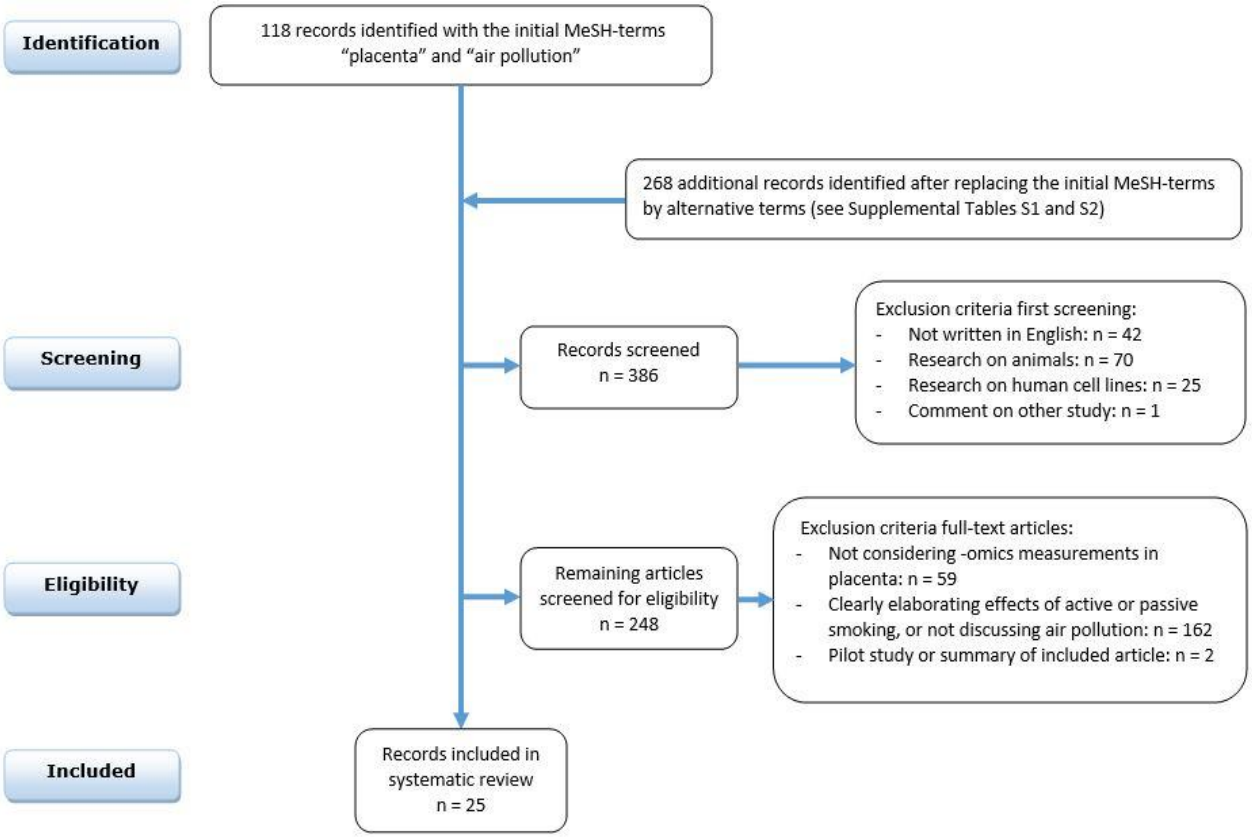

Figure 2: Flowchart of the selection protocol according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. From the 386 initially screened articles, 25 were included in this systematic review.

Replacing the MeSH-terms by alternative terms (see Supplemental Tables S1 and S2), 268 additional records could be added to the list. No new articles were identified from reference lists of other reviews and no additional information could be retrieved from grey literature. From the total of 386 articles, 42 were excluded because they were not written in English. One study was excluded since it was a comment on another research article. The abstracts of the remaining articles were scanned for eligibility based on whether they pertained to epidemiological research. We excluded 70 animal studies and 25 studies using human cell lines. Of the remaining 248 articles, 59 were excluded because they did not report -omics measurements in human placental tissue and 162 were not included since

the article only elaborated on the effects of active or passive maternal smoking and not on concomitant effects of exposure to ambient air pollution during the gestational period. One study of Topinka *et al.* (1997a) was considered a pilot study of one of the remaining articles of these authors (Topinka *et al.* 1997b), and one article of Sram *et al.* (1999) summarized the latter study, so the results of these three studies were discussed simultaneously. The inter-rater variability as determined by the Cohen's kappa analysis was 0.98 (95% confidence interval: 0.96 – 0.99), which can be regarded as a value indicating an almost perfect agreement between LJL and NDS.

Twenty-five studies (Supplemental Table S3 and Figure 1) met all the selection criteria. The publication dates of the articles ranged from August 1990 to September 2016. Six articles discussed the triple relationship involving *in utero* air pollution exposure leading to molecular changes in placental tissue, with a direct or indirect descriptive link to adverse birth outcomes and/or the development of (chronic) diseases (Clemente *et al.* 2016; Ghosh *et al.* 2013; Hincal 1986; Kingsley *et al.* 2016; Rossner *et al.* 2011; Sram *et al.* 2006). Five out of these six studies investigated a change in birth weight as an adverse outcome, three out of these five also looked at growth restriction (Hincal 1986; Kingsley *et al.* 2016; Rossner *et al.* 2011), and one article studied prematurity of the neonate as an additional detrimental birth outcome (Sram *et al.* 2006). Only one of these five studies investigated air pollution exposure during pregnancy, while looking at the associations with placental -omics markers and the development of a disease outcome later in life, namely childhood bronchitis (Ghosh *et al.* 2013).

All 25 studies were observational, conducted in an epidemiological context, and used the placenta as a biological matrix to study molecular effects of prenatal ambient air pollution exposure. Among these studies, all categories of -omics markers were covered with exception of the placental metabolome and exposome. We identified eleven studies on the genome, five on the epigenome, two on the transcriptome, five on the proteome, one study with both genomic and transcriptomic topics, and one study covered topics on both genomics and proteomics (Supplemental Figure 1). The 25

included research articles showed a bottom-up approach for all -omics categories , focusing on specific preselected targets and their association with prenatal exposure to ambient air pollution.

Twelve of the 25 articles discussed the effects of PM air pollution on placental –omics (Supplemental Figure 2). More specifically, three studies investigated PM_{10} (one study in combination with other forms of air pollution, namely PAHs, SO_2 and NOx), while nine studies investigated $PM_{2.5}$. Exposure to $PM_{2.5}$ was often studied in combination with other air pollution components, such as PAHs (three studies) and black carbon (one study). Other forms of ambient air pollution were discussed separately as well, such as NO_x (one study), and PAHs (one study). Seven articles analyzed a comparison of two groups of participants, based on their exposure to urban air pollution. Finally, four articles used proxies for air pollution exposure, such as the distance of the residence to a major road, residential traffic density, work-related air pollution exposure in maquiladoras (factories at the border between Mexico and the USA), and smoke from residential coal burning as a heating source.

4. DISCUSSION

4.1. Placental tissue in epidemiological research: advantages and disadvantages

All 25 studies that were selected for discussion in this review used placental tissue as a biological matrix for epidemiological research purposes. This temporary organ has the advantage that it can serve to evaluate biological outcomes of environmental exposures simultaneously in tissue with both maternal and fetal origin. Moreover, the sampling of placental tissue requires no invasive procedure, avoiding unnecessary potential damage to the fetus. The placenta shows to be a crucial tissue to study certain developmental processes, since it provides the necessary molecules for these mechanisms. In mice it has been shown that this organ produces serotonin at the earliest phases of pregnancy, which is an important factor in the development of the fetal central nervous system (Bonnin *et al.* 2011). Five studies discussed in this review made a link between biomolecular characteristics of the placenta and health conditions that could interfere with human development later in life, more specifically a

decrease in birth weight (Clemente *et al.* 2016; Hincal 1986; Rossner *et al.* 2011; Sram *et al.* 2006), fetal growth restriction (Hincal 1986; Rossner *et al.* 2011) or the development of bronchitis in early childhood (Ghosh *et al.* 2013). This shows that the placenta has the potential to serve as a tissue to study the link between prenatal exposures and the effects on the (mal-)development of children in early life. Apart from the different functions of umbilical cord blood and the placenta during pregnancy, several molecular differences between both matrices have been identified such as different turnover rates of mitochondrial DNA (mtDNA) (Janssen *et al.* 2012). In contrast to cord blood, which can encompass the effects of environmental exposures on the short term, the placenta can reflect the cumulative effect of prenatal exposures over the pregnancy period. In the context of the evaluation of exposure conditions on fetal development, biomolecular measurements in placental samples can be particularly useful since it has been suggested that changes in the placenta could be involved in the epigenetic regulation of fetal development, possibly to a slightly greater extent than in cord blood (Nomura *et al.* 2014).

A disadvantage of using placental tissue for research purposes is that obtaining representative sample aliquots is challenging as the placenta is composed of a heterogeneous mix of cells, blood vessels, chorionic villi, and membranes. Therefore, standardization of placental sampling is of great importance to account for the complexity of this tissue. Moreover, the sampling procedures carried out in several studies and cohorts using different protocols could introduce variability in the observed results and the conclusions drawn from this research. When comparing the sampling methods of the 25 studies included in this review, differences were identified in terms of sampling position on the placenta, the placental layers which were sampled, and the size of the tissue samples [ranging from 1-2 cm^3 (Janssen *et al.* 2012) to 50 g (Obolenskaya *et al.* 2010)]. In the context of relatively large numbers of samples or subjects under investigation in epidemiological studies and the related costs for molecular measurements, an additional disadvantage is that it is not always feasible to analyze multiple samples from the same placenta. Observational studies may consider pooling several biopsies of one placenta to further reduce sample variability. Suggestions for a more standardized protocol

have already been made by Burton *et al.* (2014), with regard to speed of sampling, aliquoting and preservation of the tissue to ensure sufficient quality of the DNA, RNA, and proteins for further analyses. These authors advice to use a standardised grid to sample each placenta at minimal four different sites, take samples of 1-2cm³, and divide these biopsies into smaller aliquots according to your -omics field of interest, and quickly snap freeze the samples after rinsing them in phosphate-buffered saline (PBS) at 4°C (Burton *et al.* 2014).

4.2. Placental -omics signatures of prenatal air pollution exposure

At delivery, the placenta is a representative source of the morphological, functional, biological, and molecular information that has been accumulated during gestation. Therefore, it is a suitable matrix for postnatal investigation of potential associations between molecular (-omics) signatures and prenatal environmental influences. Several biomolecular characteristics related to diverse toxicological exposures have already been investigated in placental tissue. Not only direct DNA damage, but also changes in -omics (genomics, epigenetics, transcriptomics, proteomics, metabolomics and exposomics) signatures can occur due to hazardous environmental exposures such as ambient air pollution (Table 1). These alterations may possibly provide early effect predictors for human health risk due to *in utero* environmental exposures (Fowler 2012). In this context, characteristic biomolecular signatures measured in humans may be considered biomarkers - which can be a chemical or its metabolite - biomolecules, or the product of an interaction between a substance and a target molecule or cell (World Health Organization 2010). The measurement of placental -omics markers can provide useful insights on gestational exposure effects, susceptibility, and disease risk of the neonate (Fowler 2012; Ryan *et al.* 2012). Despite the fact that several changes in -omics fields have been characterized in placental tissue in association with air pollution exposure, two fields - metabolomics (discussed below) and exposomics - could not be sufficiently covered in the context of this systematic review because of the lack of studies on these topics.

Table 1. -Omics categories and placental markers analyzed in association with exposure to ambient air pollution during the gestational period

-Omics category	Placental markers
Genomics	- Telomere length (Bijnens *et al.* 2015) - Mitochondrial DNA content (Clemente *et al.* 2016; Janssen *et al.* 2012) - Presence of the low activity *EPHX1* (His/His) diplotype (Ghosh *et al.* 2013) - Presence of the *CYP1A1* MspI polymorphism (Whyatt *et al.* 1998) - DNA adduct levels (Dodd-Butera *et al.* 2016; Marafie *et al.* 2000; Mumford *et al.* 1993; Reddy *et al.* 1990; Rossner *et al.* 2011; Sram *et al.* 2006; Topinka *et al.* 1997, 2009; Whyatt *et al.* 1998)
Epigenetics	- Global DNA methylation level (Janssen *et al.* 2013) - LINE-1 and AluYb8 DNA methylation levels (Kingsley *et al.* 2016) - Mitochondrial DNA methylation level (Janssen *et al.* 2015) - *LEP* promoter methylation (Saenen *et al.* 2017) - Levels of miR-21, miR-146a, miR-222, and miR-20a (Tsamou *et al.* 2016)

Transcriptomics	- Expression levels of
	  - *BDNF* (Saenen *et al.* 2015)
	  - *SYN1* (Saenen *et al.* 2015)
	  - *CYP1A1* (Whyatt *et al.* 1995, 1998)

Proteomics	- 3-NTp level (Saenen *et al.* 2016)
	- Amount of metallothionein (Sorkun *et al.* 2007)
	- GST level (Dodd-Butera *et al.* 2016)
	- Activity of
	  - AHH (Hincal 1986)
	  - Pyruvate kinase (Kedryna *et al.* 2004)
	  - GST (Dodd-Butera *et al.* 2016; Obolenskaya *et al.* 2010)
	  - ECOD (Obolenskaya *et al.* 2010)

Metabolomics	/

Abbreviations: 3-NTp, 3-nitrotyrosine; AHH, Aryl hydrocarbon hydroxylase; *BDNF*, Brain-derived neurotrophic factor; *CYP1A1*, Cytochrome (*CYP)* P450 1A1; ECOD, 7-ethoxycoumarin O-deethylase; *EPHX1*, Epoxide hydrolase 1; GST, Glutathione S-transferase; His, Histidine; *LEP*, Leptin; miR, MicroRNA; *SYN1*, Synapsin 1

The field of exposomics encompasses all the environmental exposures for an organism during its lifetime (Wild 2012). Placental exposomics have for example been studied in mothers known to be obese or diabetic at the moment of gestation (Lewis *et al.* 2013). In case of investigating the effects of *in utero* exposure to ambient air pollution one study can be cited which measured asbestos fibers as a part of the exposome in the placentas of stillborn babies (Haque *et al.* 1992). Several intermediate markers including telomere length and microRNA (miRNA) expression patterns have been studied as a proxy-effect of ambient air pollution exposure on exposomics (Martens and Nawrot 2016; Vrijens *et al.* 2015). However, the full placental exposome regarding environmental air pollution exposure is a complex entity of which the parts still need to be assembled.

4.2.1. *Genomics* (Table 2)

Direct DNA damage and damage through DNA adducts were two of the first placental markers used to evaluate the health significance of genomic insults through prenatal ambient air pollution exposure. As early as 1990, ^{32}P-postlabeling was performed in placental tissue to study the extent of DNA damage that could be inflicted by exposure to PAHs during pregnancy (Reddy *et al.* 1990). Ten years later, a similar study was published on DNA adducts in placental samples using two different techniques, i.e. nuclease P1 and butanol extraction enhancement prior to ^{32}P-postlabeling (Marafie *et al.* 2000). Both studies came to the same conclusion: the levels of placental DNA adducts did not differ significantly between women exposed to airborne PAHs by either residential wood combustion (Reddy *et al.* 1990) or pollution from oil well fires (Marafie *et al.* 2000) compared with non-exposed women. In a recent study, lack of association was also found between placental PAH-adducts and exposure to work-related air pollution at the US-Mexican border (Dodd-Butera *et al.* 2016). Mumford *et al.* (1993) came to the opposite conclusion in a study on placental DNA-adduct levels and PAH exposure during pregnancy: the adduct levels increased when mothers were exposed to smoke of coal burning during pregnancy, however, these results lack statistical confirmation. Furthermore, a study on ambient $PM_{2.5}$ and PM_{10} air pollution also did not show an association between placental DNA-adduct levels and exposure to air pollution (Sram *et al.* 2006). Hence, the consistent negative results from these

independent studies may point to a molecular effect other than the formation of DNA adducts in the placenta associated with maternal air pollution exposure. Topinka *et al.* (2009) compared placental adduct levels with those in cord blood following *in utero* exposure to PAHs and $PM_{2.5}$, and showed that the total level of DNA adducts was significantly higher in cord blood compared to placenta. Other studies in cord blood also showed positive relationships between DNA adduct levels and exposure to air pollution (Pedersen *et al.* 2009), which indicates that these hazardous airborne substances could affect DNA adduct levels in other tissues than the placenta.

Fetuses are able to adapt their mitochondrial structure and metabolism when the supply of nutrients is limited or compromised. Mitochondria are the biochemical power plants of cells providing energy through the production of adenosine-5'-triphosphate (ATP) via oxidative phosphorylation. These intracellular organelles contain multiple copies of circular DNA - mitochondrial DNA (mtDNA) - of approximately 16 kb in length which are vulnerable to reactive oxygen species (ROS) because of close proximity to the electron transport chain and inefficient DNA repair capacity(Linnane *et al.* 1989). The estimated mutation rate of mtDNA is 5-15 times higher compared to nuclear DNA (Payne *et al.* 2013). Changes in placental mtDNA content may represent a biological effect along the path linking air pollution to adverse effects on the unborn. In placental tissue of 174 mother-newborn pairs of the Belgian birth cohort ENVIR*ON*AGE, an inverse association was found between third trimester PM_{10} (and NO_2) exposure and placental mtDNA content (-17.4%, 95% CI: -31.8 to -0.1%, for an increment of 10 $\mu g/m^3$ in PM_{10} exposure; p = 0.05) (Janssen *et al.* 2012).

A similar inverse association was found In the Spanish INMA birth cohort between placental mtDNA content and gestational exposure to traffic-related NO_2 air pollution (-4.9%, 95% CI: -7.9 to -1.8% for an increment of 10 $\mu g/m^3$ in NO_2 exposure; p = 0.003) (Clemente *et al.* 2016). The discrepancy in effect-size can be explained by the very dynamic nature of placental mtDNA.

Table 2. Studies describing the associations between prenatal ambient air pollution exposure and changes in placental genomic markers

Author	Study population	Increase in analyzed air pollution component (average ± standard deviation if available)	Effect on placental -omics marker
Reddy *et al.* (1990)	4 non-smoking women exposed to wood smoke during pregnancy and 5 non-exposed controls from Massachusetts	Urban air pollution	No significant differences in DNA-adduct levels between exposed and non-exposed mothers.
Mumford *et al.* (1993)	38 placental samples from Xuan Wei (China) exposed to coal combustion smoke during pregnancy and 19 samples from controls living in Beijng, using natural gas as heating source.	Smoke from coal combustion	Total DNA-adducts detected in 52% of placentas of exposure group compared to 5.3% of the samples of the control group (no statistics performed)
Topinka *et al.* (1997)	158 mothers (113 non-smokers and 45 smokers) in two districts of the Czech Republic with different exposure levels of air pollution	Average monthly concentrations of SO_2, NOx, PAH and PM_{10} from January 1994 until January 1995	Increased levels of DNA-adducts in samples of the highly exposed regions compared to the lower exposed regions in placentas with the *GSTM1* null genotype ($p = 0.029$) No effect of NAT2 genotype on DNA adduct levels in correlation with air pollution exposure
Whyatt *et al.* (1998)	70 subjects from Krakow with higher levels of air pollution and 90 subjects from Limanowa, a less polluted city in Poland	Average annual concentration of 37 µg/m³ of ambient respirable particles in least exposed group and 78 µg/m³ in the most exposed group, in the year prior to delivery (particle size not defined)	No significant associations between PAH-adduct levels, presence of the *CYP1A1 MspI* polymorphism and exposure to air pollution.
Marafie *et al.* (2000)	40 mothers exposed to oil well fires and 180 non-exposed Kuwaiti mothers	Urban air pollution	No significantly different levels of DNA-adducts between mothers of different exposure groups
Sram *et al.* (2006)	199 subjects born between 1994 and 1995 for DNA-adduct analyses and 1013 subjects born between 2000 and 2002 for genotyping. All samples collected in two districts of the Czech Republic with different levels of air pollution	Urban air pollution	No significant associations between placental DNA-adduct levels and birth weight, and no effects of air pollution on birth weight or DNA-adduct levels identified.
Topinka *et al.* (2009)	Placentas from 79 individuals born in 2007 and 2008 in Prague (Czech Republic)	B[a]P, PAHs and $PM_{2.5}$ levels (no mean values provided)	Total DNA-adduct levels are significantly higher in cord blood compared to placental tissue ($p < 0.001$)
Rossner *et al.* (2011)	891 subjects born between 1994 and 1999 in two districts of the Czech Republic with either high or low levels of air pollution exposure	Average concentrations of PAHs and $PM_{2.5}$ for each month of pregnancy	No significant associations between 8-oxodG-adduct levels and PAH levels, but a significant increase in 8-oxodG-adduct levels with increased $PM_{2.5}$ exposure in second month of pregnancy (OR = 1.68, 95% CI: 1.28 to 2.19; $p < 0.001$)

Abbreviations: 8-oxodG, (8-oxo-2'-deoxyguanosine); B[a]P, Benzo[a]pyrene; CI, Confidence interval; *EPHX1,* Epoxide hydrolase 1; *GSTM1*, Glutathione S-transferase M1; mtDNA, Mitochondrial DNA; NAT2, N-acetyl transferase 2; NO_2, Nitrogen dioxide; NOx, Nitrogen oxides; OR, Odds ratio; PAH, Polycyclic aromatic hydrocarbon; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 µm; PM_{10}, Particulate matter with a diameter smaller than 10 µm; RTL, Relative telomere length; SO_2, Sulfur dioxide.

Table 2. (continued)

Author	Study population	Increase in analyzed air pollution component (average ± standard deviation if available)	Effect on placental -omics marker
Janssen *et al.* (2012)	174 individuals from the ENVIR*ON*AGE cohort (Belgium)	10 µg/m³ increase in PM_{10} (22.7 ± 3.7 µg/m³ for entire pregnancy)	16.1% decrease in mtDNA content in association with exposure during the last month of pregnancy (95% CI: –25.2 to –6.0%, p = 0.003)
Ghosh *et al.* (2013)	n = 793 randomly selected from children born between 1994 and 1998 in two districts of the Czech Republic	100 ng/m³ increase in PAH (63.4 ng/m³ ± 51.5 ng/m³) and a 25 µg/m³ increase in $PM_{2.5}$ (22.8 µg/m³ ± 11.9 µg/m³) for the entire pregnancy period	Significantly higher effect of both PAH (OR = 1.5, (95% CI: 1.2 to 1.9) and $PM_{2.5}$ (OR = 1.5) exposure on the development of childhood bronchitis, associated with the low activity *EPHX1* (His/His) diplotype
Bijnens *et al.* (2015)	n = 211 twins of the East Flanders Prospective Twin Study (Belgium)	Doubling of the residential distance to a major road and doubling in traffic density, as proxy for maternal traffic/air pollution exposure	5.3% increase of RTL with every doubling of the residential distance to a major road (95% CI: 1.9 to 8.9%; p=0.003) and a decrease in RTL with 4.0% for every doubling in traffic density (95% CI: −7.6 to −0.2, p = 0.04)
Clemente *et al.* (2016)	n = 376 (INMA cohort, Spain) and n = 550 (ENVIR*ON*AGE cohort, Belgium)	10 µg/m³ increase in NO_2 (25.5 ± 11.4 µg/m³ in INMA cohort and 21.1 ± 4.2 µg/m³ in ENVIR*ON*AGE cohort respectively for the entire pregnancy period)	1) ENVIR*ON*AGE cohort: 11.1% decrease in mtDNA for the second trimester (95% CI: -19.9 to -1.24%; p = 0.03) and 13.5% decrease in mtDNA for the third trimester of pregnancy (95% CI: -20.1 to -6.4%; p = 0.003). 2) INMA cohort: decrease in mtDNA content for the first (-4.1%, 95% CI: -7.1 to -1.1%; p = 0.007), second (-5.0%, 95% CI: -8.0 to -2.0%; p = 0.002) and third (-4.9%, 95% CI: -7.9 to -1.8%; p = 0.003) trimester, and for the entire pregnancy (-5.5 %, 95% CI: -8.8 to -2.1%, p = 0.002)
Dodd-Butera *et al.* (2016)	n = 54 from Tijuana, Mexico	Work-related PAH air pollution exposure from working in maquiladora factories	No significant differences in DNA-adduct levels

Abbreviations: 8-oxodG, (8-oxo-2'-deoxyguanosine); B[a]P, Benzo[a]pyrene; CI, Confidence interval; *EPHX1,* Epoxide hydrolase 1; *GSTM1*, Glutathione S-transferase M1; mtDNA, Mitochondrial DNA; NAT2, N-acetyl transferase 2; NO_2, Nitrogen dioxide; NOx, Nitrogen oxides; OR, Odds ratio; PAH, Polycyclic aromatic hydrocarbon; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 µm; PM_{10}, Particulate matter with a diameter smaller than 10 µm; RTL, Relative telomere length; SO_2, Sulfur dioxide.

383 It is known that mtDNA fluctuates under the influence of age, ethnicity, and tissue investigated, but
384 most importantly depends on oxidative stress level, cellular antioxidant capacity, type of
385 environmental factor, and dose of exposure (Castegna *et al.* 2015; Meyer *et al.* 2013). Further research
386 on this topic is essential, since alterations in placental mitochondrial function or capacity of the
387 placenta may influence fetal energy provision and development (Mayeur *et al.* 2014).

388 Telomere length predicts life span early in life (Heidinger *et al.* 2012) and captures the history
389 of inflammatory and oxidative stress effects of exposure to environmental stressors (Martens and
390 Nawrot 2016). For example, exposure-related oxidative stress and inflammation are known to
391 contribute to telomere shortening (Zhang *et al.* 2013). Bijnens *et al.* (2015) investigated changes in
392 placental telomere length in twins in correlation with traffic-related exposure. In this study, three
393 indicators of exposure were assessed, i.e. the distance from the residential address of the mother to
394 the nearest major road, traffic density within a 200 m buffer from the residence, and residential
395 greenness. The authors concluded that placental telomere length was longer in association with a
396 doubling of the residential distance to a major road (5.3%, 95% CI: 1.9 to 8.9%; p = 0.003), and shorter
397 with a doubling in traffic density (-4.0%, 95% CI: −7.6 to −0.2%; p = 0.04).

398 Other genomic factors of susceptibility in the context of health and disease are DNA
399 polymorphisms. Specific polymorphisms can cause an alteration in the metabolic capacity of cells as
400 to the degradation and/or elimination of toxic substances, such as particle-bound chemicals derived
401 from tobacco smoke or ambient air pollution. In turn, these metabolic changes could entail a new risk
402 of disease development (Bartsch *et al.* 2000). Research has focused specifically on the associations
403 between *in utero* exposure to air pollution and placental genotypes related to detoxification
404 mechanisms. One of the most important actors in this process is cytochrome P450 1A1 (*CYP1A1*), which
405 is expressed in various tissue types throughout the body including the placenta, and fulfils both a
406 detoxifying and a bioactivating role. This enzyme can bioactivate pro-carcinogenic substances such as
407 PAHs to form adducts with DNA in tissues of both the mother and child during pregnancy (Stejskalova
408 and Pavek 2011). Whyatt *et al.* (1998) focused on *in utero* air pollution exposure and its effects on

changes of placental *CYP1A1.* In the genomic category, the authors investigated the association between the homozygous ($MspI^{+/+}$) or heterozygous ($MspI^{+/-}$) presence of the *CYP1A1* MspI polymorphism in placental tissue between smokers and non-smokers within areas heavily or less polluted with PAHs. An association between the placental presence of this polymorphism and the formation of DNA adducts due to PAH air pollution could not be demonstrated (Whyatt *et al.* 1998). In addition, Sram *et al.* (2006) studied the association between *CYP1A1* polymorphisms and PAH levels in association with birth weight, but a significant effect of these placental polymorphisms on birth weight following maternal PAH exposure was not found. $PM_{2.5}$ and PM_{10} levels were also measured in this study, but no effects of these air pollution components were mentioned. Glutathione S-transferase M1 (GSTM1) and N-acetyl transferase 2 (NAT2) are two other enzymes involved in the detoxification system of cells. Studies conducted on the genotypes of these two enzymes in human placental tissue showed that both genes interact with ROS, but only the null phenotype $GSTM1^{-/-}$, unlike $GSTM1^{+/-}$ or $GSTM1^{+/+}$, was correlated with maternal exposure to SO_2, NOx and PM_{10} during pregnancy (Topinka *et al.* 1997a, 1997b). The placenta did not only prove to be a useful tissue for genomic analyses of *GST* polymorphisms in connection with air pollution exposure, but also for studying the proteomic level of placental GST activity (Obolenskaya *et al.* 2010) (see proteomics section).

4.2.2. *Epigenetics* (Table 3)

The most commonly characterized epigenetic marking process is DNA methylation, which involves the addition of a methyl group to the carbon-5 position of cytosine residues of the dinucleotide CpG. DNA methylation undergoes critical modification during early *in utero* life. After fertilization and prior to implantation, DNA methylation patterns are largely erased but are re-established by *de novo* DNA methyltransferases (DNMTs) in the blastocyst stage (Jirtle and Skinner 2007). These waves of epigenetic reprogramming likely make early embryonic development a critical period during which nutritional, environmental, and metabolic factors affect the developmental establishment of epigenetic regulation (Kelsey and Michels 2012). The placenta exhibits a different methylation profile compared to fetal somatic tissue (Magda Price *et al.* 2012) which is probably

needed to generate cells with a broad developmental potential and the correct initiation of embryonic gene expression. Indeed, the placenta shows considerable developmental plasticity which is important for adaptation to fetal and maternal signals including hormonal and environmental exposures or other responses to *in utero* conditions (Hogg *et al.* 2012). Hence, the placenta contains information on DNA methylation patterns revealing the environmental impact to which the fetus has been exposed during gestation.

An expanding body of evidence suggests that exposures to hazardous environmental factors are important determinants for altered DNA methylation-related programming during early life. These alterations can persist throughout the course of life, thereby leading to pathological conditions in adulthood. Recently, Vaiserman (2015) summarized clinical and epidemiological evidence in support of epigenetic factors that may mediate the link between early-life exposures and long-term health outcomes. Changes in DNA methylation patterns of placental tissue have been disclosed in association with adverse maternal exposures such as alcohol and tobacco smoke (Wilhelm-Benartzi *et al.* 2012), however only recently placental epigenetic signatures have been identified in association with exposure to ambient air pollution. Janssen *et al.* (2013) were the first to investigate the association between $PM_{2.5}$ exposure during pregnancy and the global DNA methylation levels in placental tissue. For the entire pregnancy period they found that an increase of 5 µg/m³ in $PM_{2.5}$ exposure correlated with a relative decrease of 2.2% in global placental DNA methylation (95% CI: -3.7 to -0.7%; p = 0.004). These findings have been replicated by Kingsley *et al.* (2016). The authors showed that pregnant mothers living closer to major roads, as a marker of traffic-related air pollution, had lower levels of placental DNA methylation in LINE-1 (-0.82%, 95% CI: −1.57 to −0.07; p = 0.03) but not AluYb8 repetitive elements, which can be regarded as surrogate markers of global DNA methylation.

457 **Table 3.** Studies describing the associations between prenatal ambient air pollution exposure and changes in placental epigenetic markers

Author	Study population	Increase in analyzed air pollution component (average ± standard deviation if available)	Effect on placental -omics marker
Janssen *et al.* (2013)	240 samples from the ENVIR*ON*AGE birth cohort (Belgium)	5 µg/m³ $PM_{2.5}$ increase (17.4 ± 3.6 µg/m³ for entire pregnancy)	Decrease in global DNA methylation for whole pregnancy (-2.2%, 95% CI: -3.7 to -0.7%; p = 0.004), first trimester (-2.4%, 95% CI: -3.6 to -1.2%; p = 0.0001) and second trimester of pregnancy (-1.5%, 95% CI: -2.7 to -0.4%; p = 0.01)
Janssen *et al.* (2015)	381 mother-newborn pairs from the ENVIR*ON*AGE birth cohort (Belgium)	3 µg/m³ (IQR) increase in $PM_{2.5}$ (16.7 ± 2.3 µg/m³)	Increased mtDNA methylation levels (0.5%, 95 % CI: 0.2 to 2.2%; p < 0.05) and decrease of mtDNA content with 15.6% (95% CI: -23.9 to -6.4%; p < 0.05)
Kingsley *et al.* (2016)	471 mother-infant pairs from the RICHS cohort (Rhode Island, USA)	Proximity of the residential distance to a major road as proxy for air pollution exposure	0.82% decrease in mean LINE-1 methylation levels (95% CI: −1.57 to −0.07; p = 0.03)
Tsamou *et al.* (2016)	210 mother-child pairs from the ENVIR*ON*AGE cohort (Belgium)	5 µg/m³ increase in $PM_{2.5}$ (16.38 ± 5.29 µg/m³ for the first trimester of pregnancy and 16.74 ± 5.82 µg/m³ for the second trimester of pregnancy)	Decreased expression of miR-21 (-33.7%, 95% CI: -53.2 to -6.2%; p = 0.02), miR-146a (-30.9%, 95% CI: -48.0 to -8.1%; p = 0.012) and miR-222 (-25.4%, 95% CI: -43.0 to -2.4%; p = 0.034) for the second trimester of pregnancy and an increased expression of miR-20a (+70.9%, 95% CI: 16.7 to 150.3%; p = 0.007) and miR-21 (+73.7%, 95% CI: 11.7 to 170.1%; p = 0.015) in the first trimester.
Saenen *et al.* (2017)	361 samples from the ENVIR*ON*AGE birth cohort (Belgium)	7.5 µg/m³ (IQR) increase in $PM_{2.5}$ (15.5 ± 4.9 µg/m³ for the second trimester)	A 1.4% decrease in *LEP* promoter methylation for the second trimester of pregnancy (95% CI: -2.7 to -0.2%; p = 0.02)

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459 Abbreviations: CI, Confidence interval; IQR, Interquartile range; *LEP,* Leptin; miR, Micro RNA; mtDNA, Mitochondrial DNA; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 µm.

Another interesting finding of the study of Janssen *et al.* (2013) was that the early gestational stage from fertilization up to and including implantation - a critical period for methylation reprogramming - is likely to represent a highly sensitive window for the effects of $PM_{2.5}$ exposure on placental DNA methylation as measured at birth. The health implications of these findings should be further investigated, since it has been shown that overall hypomethylation patterns in the placenta could be an indication of an increased risk to birth defects such as spina bifida (Zhang *et al.* 2015). Furthermore associations have been found between hypomethylation of specific promoters and adverse birth conditions such as low birth weight (Rumbajan *et al.* 2016).

Recently, attention has been drawn to the methylation pattern of a specific gene in the placenta, namely the promoter region of the leptin (*LEP*) gene. Leptin is an important hormone during pregnancy, since it plays a crucial role in fetal growth and development through its function in energy metabolism (Walsh *et al.* 2014). An interquartile range increment (IQR) of $PM_{2.5}$ exposure (7.5 $\mu g/m^3$) was associated with a 1.4% decrease in placental methylation of the *LEP* promoter region (95 % CI: -2.7 to -0.2%; p = 0.02) (Saenen *et al.* 2017). In previous research, a decrease in *LEP* methylation has been associated with gestational syndromes such as pre-eclampsia (Hogg *et al.* 2013) and impaired glucose tolerance (Bouchard *et al.* 2010). The intricate connection between *LEP* methylation, PM exposure, and disease phenotype should be explored more in depth by studying potential ailments in childhood that may arise from these placental changes.

Besides the nuclear genome, the mitochondrial genome can undergo epigenetic modifications as well. For example, maternal emotional stress during pregnancy has shown to alter gene expression patterns in placental mitochondria, which can eventually affect the temperamental development of the child in early life (Lambertini *et al.* 2015). DNA methylation in specific regions of the mitochondrial genome has been shown to substantially mediate the association between $PM_{2.5}$ exposure during gestation and placental mtDNA content which could reflect signs of mitophagy and mitochondrial death (Janssen *et al.* 2015). However, the epigenetic changes in mtDNA patterns linked to air pollution exposure have not yet been studied in the context of developmental outcomes of the newborn.

Therefore, further exploration of mitochondrial gene expression regulation by DNA methylation is of paramount importance to unravel these potentially important relationships.

A type of epigenetic mark that has not yet been investigated to a great extent in the context of prenatal air pollution exposure is microRNA (miRNA) (Vrijens *et al.* 2015). MiRNAs are endogenous, single-stranded, short non-coding RNA sequences (approximately 22 nucleotides) that regulate gene expression at the post-transcriptional level. Different cell types have both common and unique miRNA expression patterns, which can be influenced by developmental and pathologic states. The human placenta expresses a distinct subset of miRNAs, but although the functions of these placental epigenetic marks are largely unknown, recent research has revealed a functional role for miRNAs in placental biology (Gu *et al.* 2013). The presence of placental miRNAs in the maternal circulation is interesting as it could lead to the discovery of biomarkers of placental dysfunction or pregnancy-related disease (Miura *et al.* 2016). Only one study has described changes in placental miRNA expression in association with prenatal air pollution exposure. A relative decrease in the placental expression of miR-21 (-33.7%, 95% CI: -53.2 to -6.2%; p = 0.022), miR-146a (-30.9%, 95% CI: -48.0 to -8.1%; p = 0.012), and miR-222 (-25.4%, 95% CI: -43.0 to -2.4%; p = 0.034) was found in association with an increase of 5 µg/m³ $PM_{2.5}$ during the second trimester of pregnancy, whereas a positive association was described between first trimester $PM_{2.5}$ air pollution exposure and the expression of placental miR-20a (+70.9%, 95% CI: 16.7 to 150.3%; p = 0.007) and miR-21 (+73.7%, 95% CI: 11.7 to 170.1%; p = 0.015) (Tsamou *et al.* 2016). A common target of these miRNAs is the tumor suppressor phosphatase and tensin homolog (*PTEN*) which also showed an altered expression in association with $PM_{2.5}$ exposure (+59.6% per 5 µg/m³ increment, 95% CI: 26.9 to 100.7%; p < 0.0001) and, as expected, an inverse correlation with the levels of these miRNAs, since increasing levels of miRNAs are known to block the expression of their associated targets (Tsamou *et al.* 2016).

4.2.3. *Transcriptomics* (Table 4)

The area of placental transcriptomics has been poorly addressed in association with exposure to air pollution during pregnancy. One possible explanation for this is the difficulty to obtain placental

tissue aliquots with a sufficiently high RNA quality for whole transcriptome analyses, since RNA starts degrading quickly after sampling (Gallego Romero *et al.* 2014). However, various reviews have discussed the transcriptome of the placenta in the context of negative birth outcomes (Cox *et al.* 2015; Eidem *et al.* 2015). Recent research has shown that the placenta contains several distinct gene expression patterns compared with other tissues in the human body, for example when the number of slice variants and the expression levels of regulators involved in splicing are concerned (Kim *et al.* 2012). Quantitative PCR, micro-array analysis, and RNA sequencing have proven to be indispensable for the analysis of exposure effects on transcriptomic alterations, potentially leading to perturbation of developmental and biological mechanisms. Apart from their work in the genomic field, Whyatt *et al.* (1995, 1998) also investigated the effects of prenatal exposure to air pollution on the placental expression of *CYP1A1*. However, in both studies significant results concerning this gene could not be established, which was in accordance with the absence of an association in the genomic field of their research on *CYP1A1* (see section 4.2.1. Genomics).

Saenen *et al.* (2015) investigated the placental expression levels of ten genes in the brain-derived neurotrophic factor (*BDNF*) pathway in connection with PM air pollution. A 5 µg/m³ increase in residential $PM_{2.5}$ exposure of the mother during the first trimester of pregnancy was associated with a 15.9% decrease in expression of placental *BDNF* (95% CI: –28.7 to –3.2%, p = 0.015), and with a 24.3% decrease in *synapsin 1* (*SYN1*) expression (95% CI: –42.8 to –5.8%, p = 0.011) which is affected by the actions of *BDNF*. Proper functioning of this pathway in the placenta is crucial for normal fetal development, since altered *BDNF* expression in this tissue has been associated with negative birth outcomes such as fetal growth restriction (Mayeur *et al.* 2010).

Table 4. Studies describing the associations between prenatal ambient air pollution exposure and changes in placental transcriptomic markers

Author	Study population	Increase in analyzed air pollution component (average ± standard deviation if available)	Effect on placental -omics marker
Whyatt *et al.* (1995)	70 subjects from a city with higher levels of air pollution (Krakow) and 90 subjects from a less polluted city (Limanowa) in Poland	Average of 80 µg/m³ PM_{10} in highly polluted area (range, 23.4-154.2 µg/m³), no data available for Limanowa	No significant difference in *CYP1A1* mRNA levels between low and high polluted area (r = -0.4; p = 0.14).
Whyatt *et al.* (1998)	70 subjects from Krakow with higher levels of air pollution and 90 subjects from Limanowa, a less polluted city in Poland	Average annual concentration of 37 µg/m³ of ambient respirable particles in least exposed group and 78 µg/m³ in the most exposed group, in the year prior to delivery (particle size not defined)	PAH-adduct levels not significantly associated with *CYP1A1* mRNA (r = –0.10; p = 0.2)
Saenen *et al.* (2015)	90 randomly selected mother-child pairs from the ENVIR*ON*AGE birth cohort (Belgium)	5 µg/m³ increase in $PM_{2.5}$ (15.4 ± 5.4 µg/m³ for the first trimester, 17.6 ± 7.0 µg/m³ for the second trimester and 18.7 ± 6.0 µg/m³ for the third trimester of pregnancy)	15.9% decrease in placental *BDNF* expression (95% CI: –28.7 to –3.2%; p = 0.015) and a 24.3% lower expression of *SYN1* (95% CI: –42.8 to –5.8%, p = 0.011)

Abbreviations: *BDNF*, Brain-derived neurotrophic factor; CI, Confidence interval; *CYP1A1*, Cytochrome (*CYP)* P450 1A1; PAH, Polycyclic aromatic hydrocarbon; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 µm; PM_{10}, Particulate matter with a diameter smaller than 10 µm, *SYN1*, Synapsin 1.

4.2.4. *Proteomics* (Table 5)

Although proteins are the end products of the transcription of genomic sequences, there is no linear relationship between a genome and its resulting proteome because of alternative splicing and the production of non-functional proteins (Pandey and Mann 2000). Therefore, it is essential that proteomics signatures are studied as a separate -omics field which can complement the finding in other -omics categories (Pandey and Mann 2000). Proteomic characteristics have been investigated in placental tissue in association with maternal tobacco smoking habits (Machaalani *et al.* 2014) and other exposures to toxicants during pregnancy. Considerable focus has been put on proteins that play an essential role in the detoxification system of the cells. The activity of placental aryl hydrocarbon hydroxylase (AHH), which is the most important metabolizer of PAHs, was significantly higher in placentas obtained from mothers who lived in an environment exposed to urban air pollution compared with the control group (Hincal 1986). Two other cellular detoxification indicators, glutathione S-transferase (GST) and 7-ethoxycoumarin O-deethylase (ECOD), showed opposite associations with increasing air pollution levels: ECOD activity significantly increased with increasing ambient air pollution (related to industry and traffic-exhaust), while the GST activity decreased under the same conditions (Obolenskaya *et al.* 2010). Work-related exposure to another source of PAH air pollution during pregnancy, such as in the “maquiladoras” at the US-Mexican border, had no effect on the placental GST level or activity (Dodd-Butera *et al.* 2016). Detoxification processes are important for normal cellular functioning, but the maintenance of the delicate redox balance of the cell is a crucial factor as well. In controlling oxidative stress, metallothionein (MT) is an important protein for fixation and transport of metals. In a study of Sorkun *et al.* (2007), a significant increase in the amount of placental MT was observed in regions with higher levels of air pollution exposure. Another area of interest in placental proteomics addresses the energy system of the cell.

560 **Table 5.** Studies describing the associations between prenatal ambient air pollution exposure and changes in placental proteomic markers

Author	Study population	Increase in analyzed air pollution component (average ± standard deviation if available)	Effect on placental -omics marker
Hincal (1986)	152 mother-child pairs from residential Ankara and 125 mother-child pairs from the more rural areas surrounding Ankara	Urban air pollution	AHH activity was significantly higher in placental tissue of women living in Ankara compared to women of the rural areas ($p < 0.001$)
Kedryna *et al.* (2004)	15 women from Chorzow and Krakow (polluted areas) and 8 women from the Bieszczady Mountains (less polluted area) in Poland	Urban air pollution	Significant decrease in pyruvate kinase activity in more polluted areas ($p < 0.001$)
Sorkun *et al.* (2007)	Samples of 92 mothers: 33 smokers, 29 exposed to air pollution and 30 non-smokers residential in a rural area with lower levels of air pollution	Urban air pollution	Higher levels of metallothionein in group exposed to air pollution, compared to mothers living in rural area ($p = 0.013$)
Obolenskaya *et al.* (2010)	143 mothers who gave birth between 1991-1999 in polluted areas of Ukraine and Belarus, and a less polluted area in the east of Poland.	Urban air pollution	Significantly lower GST activity ($r_s = -0.27$; $p = 0.05$) and higher ECOD activity ($p < 0.05$) in highly polluted areas compared to the lower polluted areas
Saenen *et al.* (2016)	330 mother-child pairs from the ENVIR*ON*AGE birth cohort (Belgium)	3.5 µg/m³ (IQR) increase in $PM_{2.5}$ (16.1 ± 2.4 µg/m³ for whole pregnancy) and 0.36 µg/m³ (IQR) increase in BC (0.97 ± 0.28 µg/m³ for entire pregnancy)	35.0% increase in 3-NTp levels for increased $PM_{2.5}$ levels (95% CI: 13.9 to 60.0%; $p = 0.0006$) and 13.9% increase in 3-NTp levels for increased BC levels (95% CI: −0.2 to 29.9%; $p = 0.05$) during the entire pregnancy period
Dodd-Butera *et al.* (2016)	n = 54 from Tijuana, Mexico	Work-related PAH air pollution exposure from working in maquiladora factories	No significant difference in GST level ($p = 0.243$) or GST activity ($p = 0.965$) between women working in maquiladoras and women from non-exposed area

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562 Abbreviations: 3-NTp, 3-nitrotyrosine; AHH, Aryl hydrocarbon hydroxylase; BC, Black carbon; CI, Confidence interval; *CYP1A1(*2A)*, Cytochrome (*CYP)* P450 1A1 (2A); ECOD, 7-ethoxycoumarin
563 O-deethylase; *EPHX1*, Epoxide hydrolase 1; GST, Glutathione S-transferase; IQR, Interquartile range; PAH, Polycyclic aromatic hydrocarbon; $PM_{2.5}$, Particulate matter with a diameter smaller
564 than 2.5 µm.

Pyruvate kinase, an essential enzyme in the process of glycolysis, showed a significant increase of activity in placental tissue of women who lived in more polluted areas (Kedryna *et al.* 2004). The level of this protein was also increased in placental tissue of preeclampsia pregnancies (Bahr *et al.* 2014), a condition characterized by excessive inflammatory reactions in the placenta. This parallels prenatal exposure to air pollution, as inflammation is the most likely mode of action triggered by ambient air pollution (Janssen *et al.* 2012). Because glycolysis is a very important metabolic pathway of energy production in the placenta (Bloxam 1985), more attention is needed as to the effects of air pollution on this critical pathway during gestation.

Not only the intact proteins themselves, but also the products of protein degradation or modification can be measured as biomarkers of placental damage caused by detrimental influences during pregnancy. Protein damage can be caused by processes such as oxidative stress and inflammation. In this context, the tyrosine groups of proteins can be modified into 3-nitrotyrosine (3-NTp) by peroxinitrite, which is an intermediate of oxidative or nitrosative stress. Recently, a positive association was found between placental 3-NTp levels and both $PM_{2.5}$ exposure (+35.0% for a 3.5 µg/cm³ increment in $PM_{2.5}$, 95% CI: 13.9 to 60.0%; $p < 0.0006$) and BC exposure (+13.9% for a 0.36 µg/m³ increment in BC, 95% CI: −0.2 to 29.9%; $p = 0.05$) (Saenen *et al.* 2016), which is in line with recent studies on mice. These animals showed increased placental 3-NTp levels which correlated with exposure to air pollution-related diesel exhaust (Weldy *et al.* 2014).

4.2.5. *Metabolomics*

Metabolomics is a research area that deals with changes in small metabolites (lipids, amino acids, sugars, etc.) as a consequence of altered metabolism by internal or external influences (Tzoulaki *et al.* 2014). This -omics study area has been broadly addressed within several research topics concerning placental tissue. The placental metabolome of complicated pregnancies has been compared with that of normal pregnancies to investigate the molecules associated with adverse outcomes such as neural tube defects (Chi *et al.* 2014). However, the placental metabolome characteristic of prenatal exposure to ambient air pollution did not deserve any attention until now.

Metabolomic parameters linked to the effects of air pollution have been studied to a small extent in other human matrices such as umbilical cord blood plasma (e.g. oxylipins) (Martens *et al.* 2017) and lung lavage fluid (Surowiec *et al.* 2016). Since these results showed specific metabolic signatures associated with air pollution exposure, this should be an incentive to further investigate tissues such as the placenta to reveal early-life changes in metabolic pathways due to adverse exposures during pregnancy.

4.3. Triple relationship between exposure to ambient air pollution, placental biomarkers and disease development

As stated by Professor David Barker in the early 1990s, the occurrence of diseases later in life may already be initiated during fetal development as a result of detrimental *in utero* exposures and direct or indirect influences of placental involvement (Barker 1995). To fully comprehend the complexity of the fetal origin of disease, it is crucial to investigate the intricate triple relationship between exposure, molecular effect and clinical outcome (Table 6). In earlier research, morphological changes in placental tissue have been linked to chorangiosis, an adverse condition of the placenta itself, which is an indirect consequence for disease development and known to be associated with perinatal mortality and morbidity. Maternal exposure to urban ambient air pollution during the gestational period has been shown to lead to a significantly higher number of chorionic villi without a change in placental weight suggesting an increased possibility for developing chorangiosis (Akbulut *et al.* 2009). The associations between prenatal exposure to air pollution, the molecular changes in the placenta, and the consequences on developmental or disease characteristics later in life have not yet been studied extensively. Ghosh *et al.* (2013) investigated the effect of maternal gestational exposure to air pollution in relation to a specific placental genotype and the development of childhood bronchitis during the first two years of life.

Table 6. Studies describing the triple relationship between exposure to ambient air pollution during pregnancy, the associated placental -omics marker and the health outcome.

Author	Exposure measured in ambient air	Placental measurement	Disease / health condition
Hincal F. (1986)	Urban air pollution	Placental AHH activity	Low birth weight and shorter birth length
Sram *et al.* (2006)	$PM_{2.5}$, PM_{10}	DNA adducts and *GSTM1, GSTP1, GSTT1, CYP1A1*2A* and *CYP1A1*2C* genotypes	Low birth weight and prematurity
Rossner *et al.* (2011)	PAHs, $PM_{2.5}$	SNP analysis for 95 genes and measurement of 8-oxodG adducts	Low birth weight and intrauterine growth restriction
Ghosh *et al.* (2013)	PAH, $PM_{2.5}$	Six SNPs (*GSTM1, GSTP1, GSTT1, CYP1A1 MspI, EPHX1* exon 3 and 4) and one *EPHX1* diplotype	Acute bronchitis in early childhood
Clemente *et al.* (2016)	NO_2	mtDNA content	Low birth weight
Kingsley *et al.* (2016)	Residential proximity to a major road	DNA methylation (LINE and AluYb8 elements)	Low birth weight and small for gestational age

Abbreviations: 8-oxodG, (8-oxo-2'-deoxyguanosine); AHH, Aryl hydrocarbon hydroxylase; *CYP1A1(*2A)*, Cytochrome (*CYP)* P450 1A1 (2A); *EPHX1*, Epoxide hydrolase 1; GSTM1, Glutathione S-transferase M1; GSTP1, Glutathione S-transferase P1; GSTT1, Glutathione S-transferase T1; His, Histidine; *LEP*, Leptin; miR, MicroRNA; mtDNA, Mitochondrial DNA; *MspI*, Substitution of isoleucine to valine in the 3' non-coding region of *CYP1A1,* NO_2, Nitrogen dioxide; PAH, Polycyclic aromatic hydrocarbon; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 μm; PM_{10}, Particulate matter with a diameter smaller than 10 μm; SNP, Single nucleotide polymorphism.

A significant relationship was identified between the development of childhood bronchitis and the presence of a low activity *EPHX1* polymorphism in the placenta with increased exposure to PAH and $PM_{2.5}$. These authors were thereby the first to identify a link between prenatal exposure to air pollution, a placental -omics marker and disease development.

Several studies have focused on placental -omics signatures and adverse birth outcomes such as reduced birth weight, intrauterine growth restriction, small for gestational age, and prematurity. In earlier studies, no significant associations were found between ambient air pollution exposure, birth weight and either the levels of DNA adducts in the placenta (Sram *et al.* 2006) or the activity of aryl hydrocarbon hydroxylase in placenta (AHH) (Hincal 1986), although a link was shown between air pollution exposure and AHH activity (see proteomics section). Three more recent studies found significant negative correlations between birth weight and the levels of NO_2 (Clemente *et al.* 2016), $PM_{2.5}$ and PAHs (Rossner *et al.* 2011), and the residential proximity to a major road (Kingsley *et al.* 2016). However, Rossner *et al.* (2011) concluded that the levels of 8-oxo-deoxyguanosine (8-oxodG) DNA adducts, a marker of direct oxidative DNA damage, measured in placental tissue were only correlated with $PM_{2.5}$ levels but not with birth weight. The same conclusion was reached for the association between residential distance to a major road and LINE element methylation: LINE methylation levels were only negatively associated with a distance to a major road, but no link with birth weight could be identified (Kingsley *et al.* 2016). The only mediation analysis to investigate the triple association between air pollution exposure, placental –omics and disease development was performed with data of the INMA birth cohort (Clemente *et al.* 2016). This analysis showed that 10% of the association between a 10 $\mu g/m^3$ increase in NO_2 exposure during pregnancy and reduced birth weight could be mediated by a decrease in mitochondrial DNA levels. The results of these three studies could suggest that prenatal exposure to air pollution might exert its effects on birth outcomes by altering more subtle regulations such as those of the placental energy system and not by direct damage to the placental DNA. However, since a broad array of possible molecular factors could be responsible for the link between *in utero* air pollution exposure and an effect on disease development later in life,

integration of results on all -omics fields and the potential associations with prenatal exposure to ambient air pollution and childhood development should be prioritized in future research. This could eventually aid in the understanding of the complex etiology of adult diseases.

The strength of this review is that this is, to our knowledge, the first descriptive work to summarize and discuss the current knowledge on all placental -omics signatures that have been analyzed in association with prenatal air pollution exposure. This article has identified the current areas with the greatest gaps of knowledge which need to be addressed in future research and can therefore be a base to guide placental -omics research. A limitation in this review is the heterogeneity of the study designs of the 25 discussed articles. Differences were apparent in the approaches used to obtain the –omics data, the placental sampling protocols, and in both exposure assessment as well as the definition of the specific exposure windows. For these reasons a formal meta-analysis to combine the study results was not possible.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Exposure to air pollution in daily life is unavoidable. A crucial time window of exposure in the course of human life is fetal development. The feto-placental unit is subjected to maternal conditions and exposures that can adversely affect -omics characteristics of the placenta. Eventually, these placental changes could potentially lead to alterations in metabolic capacities of the fetus and an increased risk of disease development later in life. This systematic review shows that the placenta is a suitable tissue to investigate the effects of prenatal exposure to ambient air pollution by examining -omics biomarkers. The placenta is a temporary organ that reflects various exposures throughout pregnancy. Important in this branch of research is to have a representation of the effects of these exposures on the whole placenta. Since not only inter-, but also intra-placental differences should always be taken into account, researchers should try to find a consensus on a unified, standardized method to work

with a pooled sample of each placenta. Especially in -omics research, placental sampling is crucial because of the fragility of DNA, protein and especially RNA structures. Therefore, standardization and communication about sampling methods is crucial in -omics research. In this way, results over different cohorts could be more easily compared and discussed.

At this point in time, this systematic review shows that some -omics fields are more represented than others in the research on the effects of prenatal exposure to air pollution on placental biomarkers. The most focus has been put on the presence of placental DNA adducts, although only a minority of studies found significant effects of air pollution exposure on these biomarkers. Therefore, more attention should be put on other, more promising -omics fields such as epigenetics, transcriptomics, and proteomics. At present, several placental -omics markers have been suggested that could provide a better insight on how the consequences of exposure to ambient air pollution are manifested during pregnancy. However, most studies only focus on specific components of molecular systems and pathways. Integrating a top-down approach is crucial for epigenomics, transcriptomics and proteomics for a full understanding of the array of molecular changes that result from detrimental environmental exposures. More studies containing large qualitative datasets should combine candidate -omics markers with the exploration of entire metabolic pathways in the full genome, transcriptome, epigenome, proteome or metabolome. Eventually, this should provide a complete molecular signature of key players describing the effects of prenatal environmental exposure on placental functioning and fetal (disease) development.

Two -omics fields, - metabolomics and exposomics -, could not be sufficiently covered in the context of this systematic review because of the current paucity of such studies. More attention should be put on these fields to further expand the knowledge on placental biomolecular signatures of prenatal air pollution exposure. In general, the effects of detrimental exposures on placental molecular changes and the subsequent effects on the programming of pathologies later in life are rather scarcely documented. Therefore, future research should focus more on integrative projects such as the

epigenome-wide association studies (EWAS) to identify key molecular regulators in the etiology of disease processes. Also, more longitudinal follow-up research is needed to identify and clarify the triple link between *in utero* exposure to ambient air pollution, changes in placental -omics categories, and disease initiation/progression later in life. Two projects that are already integrating several hazardous exposures such as ambient air pollution, the molecular signatures of these exposures in several tissues and the health effects on newborns, children and adults are the Human Early-Life Exposome (HELIX) project (Vrijheid *et al.* 2014) and the EXPOsOMICS project (Vineis *et al.* 2016). In conclusion, future integrative long-term research looks promising in elucidating the underlying placental mechanisms that potentially influence disease development later in life, as a consequence of gestational air pollution exposure.

6. DECLARATIONS

6.1. COMPETING FINANCIAL INTERESTS DECLARATION

All authors declare they do not have any competing financial interests.

6.2. FUNDING

This work was funded by Leefmilieu Brussel (ref. R-7598, 2016/53753), the European Research Council (ERC-2012-StG 310898), and the Flemish Scientific Fund (FWO, N1516112/G.0.873.11.N.10). Leen J. Luyten was financed by the Bijzonder Onderzoeksfonds (BOF) partnership between the UHasselt (Diepenbeek, Belgium) and the UNamur (Namur, Belgium) (BOF15DOCNA01). Bram G. Janssen and Karen Vrijens are postdoctoral fellows of the Research Foundation Flanders (FWO) and Florence Debacq-Chainiaux is a Research Associate of the FNRS, Belgium.

6.3. ACKNOWLEDGEMENTS

The authors thank graphic design studio Reejeel for the creation of Figure 1 of this systematic review.

6.4. AUTHORS' CONTRIBUTIONS

Selection criteria for the in- or exclusion of articles were determined by LJL, NDS, and TSN. The literature search was performed by LJL and NDS, and TSN was consulted if any discrepancies remained between these two researchers. LJL wrote the first draft of the systematic review, with contributions from NDS (suggestions on section "4.1. Placental tissue in epidemiological research: advantages and disadvantages"), BGJ (section "4.2.2. Epigenetics" on (mt)DNA methylation) and KV (section "4.2.2. Epigenetics" on miRNA). HAR put special attention to the construction and finalization of the manuscript. All authors read and discussed the final version of the manuscript and approved it before submission.

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