

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

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20 Running title: Placental -omics and air pollution

21 **ABSTRACT**

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

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

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

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

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

45

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

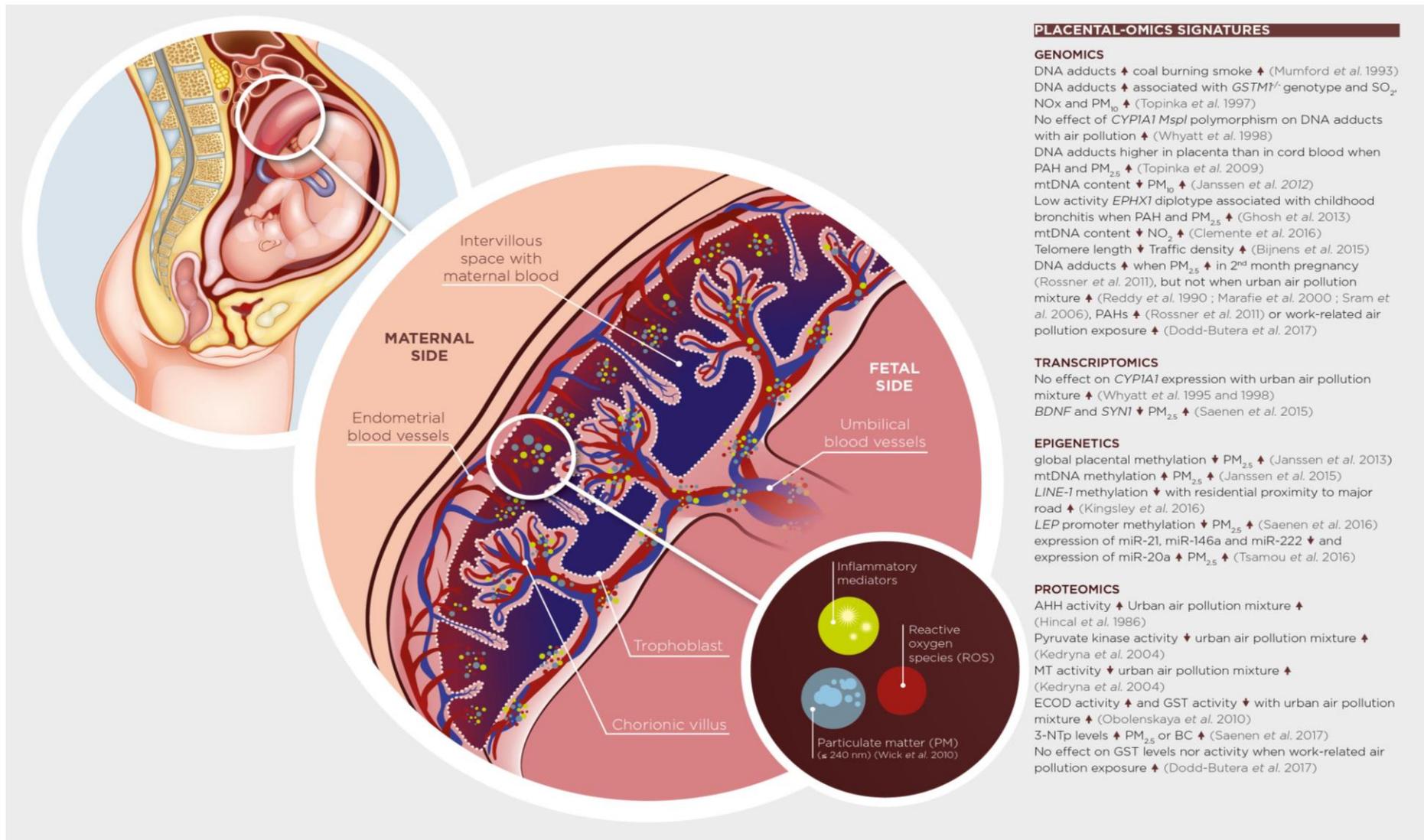
47 **ABBREVIATIONS**

48	3-NTp:	3-nitrotyrosine
49	8-oxodG:	(8-oxo-2'-deoxyguanosine)
50	AHH:	Aryl hydrocarbon hydroxylase
51	BC:	Black carbon
52	BDNF:	Brain-derived neurotrophic factor
53	CI:	Confidence interval
54	CYP1A1:	Cytochrome P450 1A1
55	ECOD:	7-ethoxycoumarin O-deethylase
56	GST:	Glutathione S-transferase
57	GSTM1:	Glutathione S-transferase M1
58	LEP:	Leptin
59	miRNA:	MicroRNA
60	MT:	Metallothionein
61	mtDNA:	Mitochondrial DNA
62	NAT2:	N-acetyl transferase 2
63	NO <sub>2</sub> :	Nitrogen dioxide
64	PAH:	Polycyclic aromatic hydrocarbon
65	PECO:	Population, Exposure, Comparator, and Outcome elements
66	PM:	Particulate matter
67	PM <sub>2.5</sub> :	Particulate matter with a diameter smaller than 2.5 μm
68	PM <sub>10</sub> :	Particulate matter with a diameter smaller than 10 μm
69	SO <sub>2</sub> :	Sulfur dioxide
70	SYN1:	Synapsin 1

## 71 1. INTRODUCTION

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

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



93

94 **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.  
 95 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  
 96 *in utero* ambient air pollution.

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

109 Intrauterine exposure to pollutants can lead to altered metabolic functions that may be  
110 detrimental for fetal development. For example, the embryonic brain has a great plasticity and its  
111 development depends on, and can be influenced by, various environmental factors (Buss *et al.* 2013).  
112 The etiology of diseases in adulthood may have a fetal origin and may be attributed to the effects of  
113 adverse environmental exposures *in utero*. This causality concept is known as the Barker hypothesis or  
114 the Developmental Origins of Health and Disease (DOHaD). Professor David Barker was the first to  
115 recognize this potential link when he became concerned about the association between malnutrition  
116 during pregnancy and the development of coronary heart disease in adult life (Barker 1995). Since  
117 then, many implications of this hypothesis have been reported (Deng *et al.* 2016, 2017; Lu *et al.* 2017).  
118 Adverse environmental exposures during pregnancy already identified in this context are active and  
119 passive cigarette smoke (Mund *et al.* 2013), and exposure to ambient air pollution [including nitrogen  
120 dioxide (NO<sub>2</sub>) (Ballester *et al.* 2010), polycyclic aromatic hydrocarbons (PAH) (Jedrychowski *et al.* 2015),  
121 and particulate matter (PM) (Rappazzo *et al.* 2014)]. Particles with a diameter smaller than 500 nm are  
122 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.

## 137 2. MATERIALS AND METHODS

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

- 144 • “Population”: human. In this article we focused on research conducted in an epidemiological  
145 context, thus not including research on human cell lines.
- 146 • “Exposure”: ambient air pollution [including particulate matter with particles smaller than 2.5  
147  $\mu\text{m}$  (PM<sub>2.5</sub>), particulate matter with particles smaller than 10  $\mu\text{m}$  (PM<sub>10</sub>), ultrafine particles,  
148 black carbon (BC), derivatives of nitrogen oxide (NO<sub>x</sub>), and polycyclic aromatic hydrocarbons  
149 (PAHs)]. We defined ambient air pollution as a mixture of indoor and outdoor pollutants, in  
150 both solid and gaseous form, and we excluded direct (maternal) or indirect (environmental)  
151 exposure to tobacco smoke from this concept.
- 152 • “Comparator”: in this review were included both studies in which comparisons are made  
153 between groups exposed to either a higher or a lower concentration of air pollution, as well  
154 as studies with a continuous exposure scale.
- 155 • “Outcomes”: placental -omics biomarkers and, if discussed, disease development or the  
156 development of adverse birth outcomes.

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

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

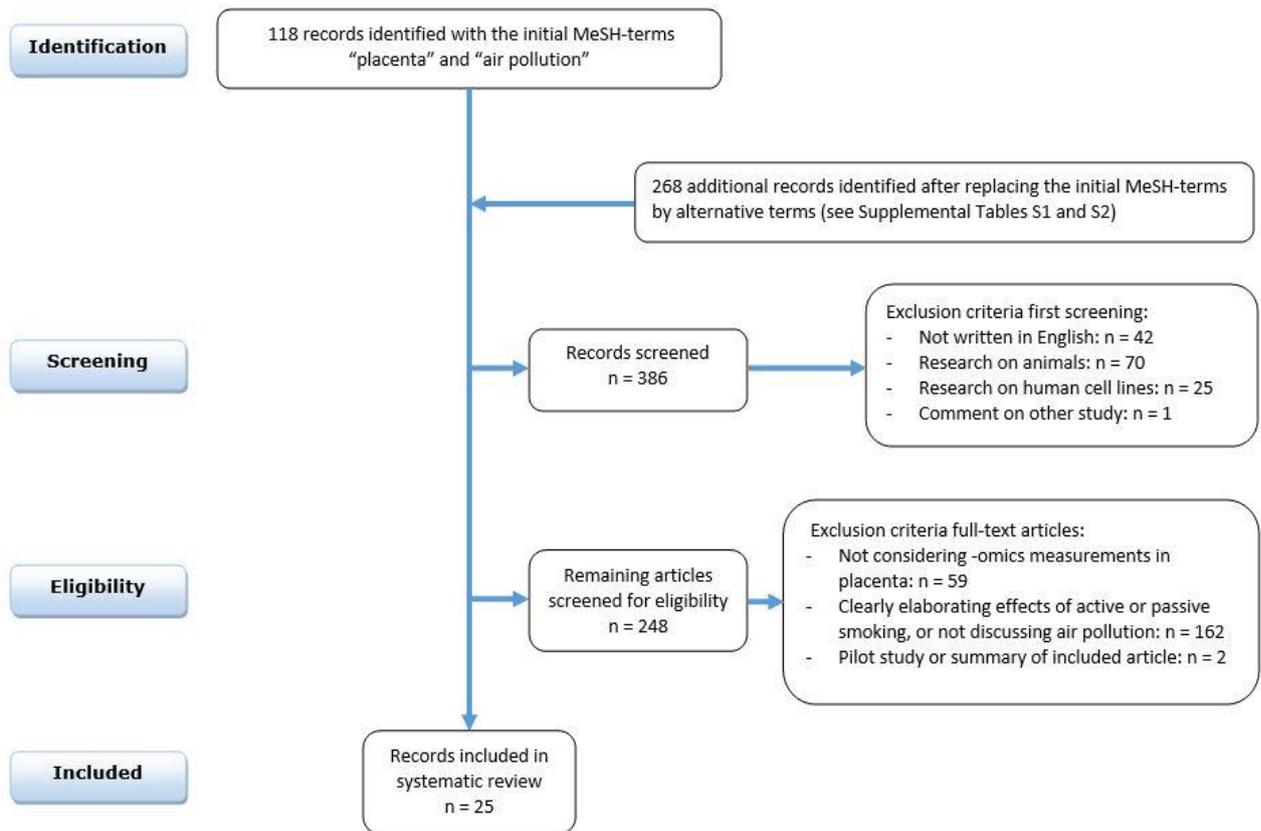
188 those studying exclusively the effect of maternal active and/or passive smoking during pregnancy on  
189 placental -omics signatures or fetal health. These articles were excluded because air pollution is a  
190 complex mixture that takes into account the effects of various sources, such as traffic- 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.

202 **3. RESULTS**

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

204

205



206

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

209

210 Replacing the MeSH-terms by alternative terms (see Supplemental Tables S1 and S2), 268 additional

211 records could be added to the list. No new articles were identified from reference lists of other reviews

212 and no additional information could be retrieved from grey literature. From the total of 386 articles,

213 42 were excluded because they were not written in English. One study was excluded since it was a

214 comment on another research article. The abstracts of the remaining articles were scanned for

215 eligibility based on whether they pertained to epidemiological research. We excluded 70 animal

216 studies and 25 studies using human cell lines. Of the remaining 248 articles, 59 were excluded because

217 they did not report -omics measurements in human placental tissue and 162 were not included since

218 the article only elaborated on the effects of active or passive maternal smoking and not on concomitant  
219 effects of exposure to ambient air pollution during the gestational period. One study of Topinka *et al.*  
220 (1997a) was considered a pilot study of one of the remaining articles of these authors (Topinka *et al.*  
221 1997b), and one article of Sram *et al.* (1999) summarized the latter study, so the results of these three  
222 studies were discussed simultaneously. The inter-rater variability as determined by the Cohen's kappa  
223 analysis was 0.98 (95% confidence interval: 0.96 – 0.99), which can be regarded as a value indicating  
224 an almost perfect agreement between LJL and NDS.

225           Twenty-five studies (Supplemental Table S3 and Figure 1) met all the selection criteria. The  
226 publication dates of the articles ranged from August 1990 to September 2016. Six articles discussed  
227 the triple relationship involving *in utero* air pollution exposure leading to molecular changes in  
228 placental tissue, with a direct or indirect descriptive link to adverse birth outcomes and/or the  
229 development of (chronic) diseases (Clemente *et al.* 2016; Ghosh *et al.* 2013; Hincal 1986; Kingsley *et*  
230 *al.* 2016; Rossner *et al.* 2011; Sram *et al.* 2006). Five out of these six studies investigated a change in  
231 birth weight as an adverse outcome, three out of these five also looked at growth restriction (Hincal  
232 1986; Kingsley *et al.* 2016; Rossner *et al.* 2011), and one article studied prematurity of the neonate as  
233 an additional detrimental birth outcome (Sram *et al.* 2006). Only one of these five studies investigated  
234 air pollution exposure during pregnancy, while looking at the associations with placental -omics  
235 markers and the development of a disease outcome later in life, namely childhood bronchitis (Ghosh  
236 *et al.* 2013).

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

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

245 Twelve of the 25 articles discussed the effects of PM air pollution on placental –omics  
246 (Supplemental Figure 2). More specifically, three studies investigated PM<sub>10</sub> (one study in combination  
247 with other forms of air pollution, namely PAHs, SO<sub>2</sub> and NO<sub>x</sub>), while nine studies investigated PM<sub>2.5</sub>.  
248 Exposure to PM<sub>2.5</sub> was often studied in combination with other air pollution components, such as PAHs  
249 (three studies) and black carbon (one study). Other forms of ambient air pollution were discussed  
250 separately as well, such as NO<sub>x</sub> (one study), and PAHs (one study). Seven articles analyzed a comparison  
251 of two groups of participants, based on their exposure to urban air pollution. Finally, four articles used  
252 proxies for air pollution exposure, such as the distance of the residence to a major road, residential  
253 traffic density, work-related air pollution exposure in maquiladoras (factories at the border between  
254 Mexico and the USA), and smoke from residential coal burning as a heating source.

255

## 256 **4. DISCUSSION**

### 257 **4.1. Placental tissue in epidemiological research: advantages and disadvantages**

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

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

281           A disadvantage of using placental tissue for research purposes is that obtaining representative  
282 sample aliquots is challenging as the placenta is composed of a heterogeneous mix of cells, blood  
283 vessels, chorionic villi, and membranes. Therefore, standardization of placental sampling is of great  
284 importance to account for the complexity of this tissue. Moreover, the sampling procedures carried  
285 out in several studies and cohorts using different protocols could introduce variability in the observed  
286 results and the conclusions drawn from this research. When comparing the sampling methods of the  
287 25 studies included in this review, differences were identified in terms of sampling position on the  
288 placenta, the placental layers which were sampled, and the size of the tissue samples [ranging from 1-  
289 2 cm<sup>3</sup> (Janssen *et al.* 2012) to 50 g (Obolenskaya *et al.* 2010)]. In the context of relatively large numbers  
290 of samples or subjects under investigation in epidemiological studies and the related costs for  
291 molecular measurements, an additional disadvantage is that it is not always feasible to analyze  
292 multiple samples from the same placenta. Observational studies may consider pooling several biopsies  
293 of one placenta to further reduce sample variability. Suggestions for a more standardized protocol

294 have already been made by Burton *et al.* (2014), with regard to speed of sampling, aliquoting and  
295 preservation of the tissue to ensure sufficient quality of the DNA, RNA, and proteins for further  
296 analyses. These authors advice to use a standardised grid to sample each placenta at minimal four  
297 different sites, take samples of 1-2cm<sup>3</sup>, and divide these biopsies into smaller aliquots according to  
298 your -omics field of interest, and quickly snap freeze the samples after rinsing them in phosphate-  
299 buffered saline (PBS) at 4°C (Burton *et al.* 2014).

300

#### 301 4.2. Placental -omics signatures of prenatal air pollution exposure

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

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

<b>-Omics category</b>	<b>Placental markers</b>
<b>Genomics</b>	<ul style="list-style-type: none"> <li>- Telomere length (Bijnens <i>et al.</i> 2015)</li> <li>- Mitochondrial DNA content (Clemente <i>et al.</i> 2016; Janssen <i>et al.</i> 2012)</li> <li>- Presence of the low activity <i>EPHX1</i> (His/His) diplotype (Ghosh <i>et al.</i> 2013)</li> <li>- Presence of the <i>CYP1A1</i> MspI polymorphism (Whyatt <i>et al.</i> 1998)</li> <li>- DNA adduct levels (Dodd-Butera <i>et al.</i> 2016; Marafie <i>et al.</i> 2000; Mumford <i>et al.</i> 1993; Reddy <i>et al.</i> 1990; Rossner <i>et al.</i> 2011; Sram <i>et al.</i> 2006; Topinka <i>et al.</i> 1997, 2009; Whyatt <i>et al.</i> 1998)</li> </ul>
<b>Epigenetics</b>	<ul style="list-style-type: none"> <li>- Global DNA methylation level (Janssen <i>et al.</i> 2013)</li> <li>- LINE-1 and AluYb8 DNA methylation levels (Kingsley <i>et al.</i> 2016)</li> <li>- Mitochondrial DNA methylation level (Janssen <i>et al.</i> 2015)</li> <li>- <i>LEP</i> promoter methylation (Saenen <i>et al.</i> 2017)</li> <li>- Levels of miR-21, miR-146a, miR-222, and miR-20a (Tsamou <i>et al.</i> 2016)</li> </ul>
<b>Transcriptomics</b>	<ul style="list-style-type: none"> <li>- Expression levels of               <ul style="list-style-type: none"> <li>- <i>BDNF</i> (Saenen <i>et al.</i> 2015)</li> <li>- <i>SYN1</i> (Saenen <i>et al.</i> 2015)</li> <li>- <i>CYP1A1</i> (Whyatt <i>et al.</i> 1995, 1998)</li> </ul> </li> </ul>
<b>Proteomics</b>	<ul style="list-style-type: none"> <li>- 3-NTP level (Saenen <i>et al.</i> 2016)</li> <li>- Amount of metallothionein (Sorkun <i>et al.</i> 2007)</li> <li>- GST level (Dodd-Butera <i>et al.</i> 2016)</li> <li>- Activity of               <ul style="list-style-type: none"> <li>- AHH (Hincal 1986)</li> <li>- Pyruvate kinase (Kedryna <i>et al.</i> 2004)</li> <li>- GST (Dodd-Butera <i>et al.</i> 2016; Obolenskaya <i>et al.</i> 2010)</li> <li>- ECOD (Obolenskaya <i>et al.</i> 2010)</li> </ul> </li> </ul>
<b>Metabolomics</b>	/

320

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

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

#### 333 **4.2.1. Genomics (Table 2)**

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

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

357           Fetuses are able to adapt their mitochondrial structure and metabolism when the supply of  
358 nutrients is limited or compromised. Mitochondria are the biochemical power plants of cells providing  
359 energy through the production of adenosine-5'-triphosphate (ATP) via oxidative phosphorylation.  
360 These intracellular organelles contain multiple copies of circular DNA - mitochondrial DNA (mtDNA) -  
361 of approximately 16 kb in length which are vulnerable to reactive oxygen species (ROS) because of  
362 close proximity to the electron transport chain and inefficient DNA repair capacity(Linnane *et al.* 1989).  
363 The estimated mutation rate of mtDNA is 5-15 times higher compared to nuclear DNA (Payne *et al.*  
364 2013). Changes in placental mtDNA content may represent a biological effect along the path linking air  
365 pollution to adverse effects on the unborn. In placental tissue of 174 mother-newborn pairs of the  
366 Belgian birth cohort ENVIRONAGE, an inverse association was found between third trimester PM<sub>10</sub>  
367 (and NO<sub>2</sub>) exposure and placental mtDNA content (-17.4%, 95% CI: -31.8 to -0.1%, for an increment of  
368 10 µg/m<sup>3</sup> in PM<sub>10</sub> exposure; p = 0.05) (Janssen *et al.* 2012).

369           A similar inverse association was found In the Spanish INMA birth cohort between placental  
370 mtDNA content and gestational exposure to traffic-related NO<sub>2</sub> air pollution (-4.9%, 95% CI: -7.9 to -  
371 1.8% for an increment of 10 µg/m<sup>3</sup> in NO<sub>2</sub> exposure; p = 0.003) (Clemente *et al.* 2016). The discrepancy  
372 in effect-size can be explained by the very dynamic nature of placental mtDNA.

373 **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
<b>Reddy et al. (1990)</b>	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.
<b>Mumford et al. (1993)</b>	38 placental samples from Xuan Wei (China) exposed to coal combustion smoke during pregnancy and 19 samples from controls living in Beijing, 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)
<b>Topinka et al. (1997)</b>	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 <sub>2</sub> , NO <sub>x</sub> , PAH and PM <sub>10</sub> 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 <i>GSTM1</i> null genotype (p = 0.029) No effect of NAT2 genotype on DNA adduct levels in correlation with air pollution exposure
<b>Whyatt et al. (1998)</b>	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/m <sup>3</sup> of ambient respirable particles in least exposed group and 78 µg/m <sup>3</sup> 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 <i>CYP1A1 MspI</i> polymorphism and exposure to air pollution.
<b>Marafie et al. (2000)</b>	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
<b>Sram et al. (2006)</b>	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.
<b>Topinka et al. (2009)</b>	Placentas from 79 individuals born in 2007 and 2008 in Prague (Czech Republic)	B[a]P, PAHs and PM <sub>2.5</sub> levels (no mean values provided)	Total DNA-adduct levels are significantly higher in cord blood compared to placental tissue (p < 0.001)
<b>Rossner et al. (2011)</b>	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 <sub>2.5</sub> 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 <sub>2.5</sub> exposure in second month of pregnancy (OR = 1.68, 95% CI: 1.28 to 2.19; p < 0.001)

374

375 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  
 376 DNA; NAT2, N-acetyl transferase 2; NO<sub>2</sub>, Nitrogen dioxide; NO<sub>x</sub>, Nitrogen oxides; OR, Odds ratio; PAH, Polycyclic aromatic hydrocarbon; PM<sub>2.5</sub>, Particulate matter with a diameter smaller than  
 377 2.5 µm; PM<sub>10</sub>, Particulate matter with a diameter smaller than 10 µm; RTL, Relative telomere length; SO<sub>2</sub>, Sulfur dioxide.

378 **Table 2. (continued)**

Author	Study population	Increase in analyzed air pollution component (average $\pm$ standard deviation if available)	Effect on placental -omics marker
<b>Janssen <i>et al.</i> (2012)</b>	174 individuals from the ENVIRONAGE cohort (Belgium)	10 $\mu\text{g}/\text{m}^3$ increase in $\text{PM}_{10}$ ( $22.7 \pm 3.7 \mu\text{g}/\text{m}^3$ 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%, $p = 0.003$ )
<b>Ghosh <i>et al.</i> (2013)</b>	n = 793 randomly selected from children born between 1994 and 1998 in two districts of the Czech Republic	100 $\text{ng}/\text{m}^3$ increase in PAH ( $63.4 \text{ ng}/\text{m}^3 \pm 51.5 \text{ ng}/\text{m}^3$ ) and a 25 $\mu\text{g}/\text{m}^3$ increase in $\text{PM}_{2.5}$ ( $22.8 \mu\text{g}/\text{m}^3 \pm 11.9 \mu\text{g}/\text{m}^3$ ) for the entire pregnancy period	Significantly higher effect of both PAH (OR = 1.5, (95% CI: 1.2 to 1.9) and $\text{PM}_{2.5}$ (OR = 1.5) exposure on the development of childhood bronchitis, associated with the low activity <i>EPHX1</i> (His/His) diplotype
<b>Bijnens <i>et al.</i> (2015)</b>	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, $p = 0.04$ )
<b>Clemente <i>et al.</i> (2016)</b>	n = 376 (INMA cohort, Spain) and n = 550 (ENVIRONAGE cohort, Belgium)	10 $\mu\text{g}/\text{m}^3$ increase in $\text{NO}_2$ ( $25.5 \pm 11.4 \mu\text{g}/\text{m}^3$ in INMA cohort and $21.1 \pm 4.2 \mu\text{g}/\text{m}^3$ in ENVIRONAGE cohort respectively for the entire pregnancy period)	1) ENVIRONAGE 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%, $p = 0.002$ )
<b>Dodd-Butera <i>et al.</i> (2016)</b>	n = 54 from Tijuana, Mexico	Work-related PAH air pollution exposure from working in maquiladora factories	No significant differences in DNA-adduct levels

379

380 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  
381 DNA; NAT2, N-acetyl transferase 2;  $\text{NO}_2$ , Nitrogen dioxide;  $\text{NO}_x$ , Nitrogen oxides; OR, Odds ratio; PAH, Polycyclic aromatic hydrocarbon;  $\text{PM}_{2.5}$ , Particulate matter with a diameter smaller than  
382 2.5  $\mu\text{m}$ ;  $\text{PM}_{10}$ , Particulate matter with a diameter smaller than 10  $\mu\text{m}$ ; RTL, Relative telomere length;  $\text{SO}_2$ , 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). Bijmens *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

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

#### 425 **4.2.2. Epigenetics (Table 3)**

426 The most commonly characterized epigenetic marking process is DNA methylation, which  
427 involves the addition of a methyl group to the carbon-5 position of cytosine residues of the  
428 dinucleotide CpG. DNA methylation undergoes critical modification during early *in utero* life. After  
429 fertilization and prior to implantation, DNA methylation patterns are largely erased but are re-  
430 established by *de novo* DNA methyltransferases (DNMTs) in the blastocyst stage (Jirtle and Skinner  
431 2007). These waves of epigenetic reprogramming likely make early embryonic development a critical  
432 period during which nutritional, environmental, and metabolic factors affect the developmental  
433 establishment of epigenetic regulation (Kelsey and Michels 2012). The placenta exhibits a different  
434 methylation profile compared to fetal somatic tissue (Magda Price *et al.* 2012) which is probably

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

441           An expanding body of evidence suggests that exposures to hazardous environmental factors  
442 are important determinants for altered DNA methylation-related programming during early life. These  
443 alterations can persist throughout the course of life, thereby leading to pathological conditions in  
444 adulthood. Recently, Vaiserman (2015) summarized clinical and epidemiological evidence in support  
445 of epigenetic factors that may mediate the link between early-life exposures and long-term health  
446 outcomes. Changes in DNA methylation patterns of placental tissue have been disclosed in association  
447 with adverse maternal exposures such as alcohol and tobacco smoke (Wilhelm-Benartzi *et al.* 2012),  
448 however only recently placental epigenetic signatures have been identified in association with  
449 exposure to ambient air pollution. Janssen *et al.* (2013) were the first to investigate the association  
450 between PM<sub>2.5</sub> exposure during pregnancy and the global DNA methylation levels in placental tissue.  
451 For the entire pregnancy period they found that an increase of 5 µg/m<sup>3</sup> in PM<sub>2.5</sub> exposure correlated  
452 with a relative decrease of 2.2% in global placental DNA methylation (95% CI: -3.7 to -0.7%; p = 0.004).  
453 These findings have been replicated by Kingsley *et al.* (2016). The authors showed that pregnant  
454 mothers living closer to major roads, as a marker of traffic-related air pollution, had lower levels of  
455 placental DNA methylation in LINE-1 (-0.82%, 95% CI: -1.57 to -0.07; p = 0.03) but not AluYb8 repetitive  
456 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 $\pm$ standard deviation if available)	Effect on placental -omics marker
<b>Janssen <i>et al.</i> (2013)</b>	240 samples from the ENVIRONAGE birth cohort (Belgium)	5 $\mu\text{g}/\text{m}^3$ PM <sub>2.5</sub> increase (17.4 $\pm$ 3.6 $\mu\text{g}/\text{m}^3$ 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)
<b>Janssen <i>et al.</i> (2015)</b>	381 mother-newborn pairs from the ENVIRONAGE birth cohort (Belgium)	3 $\mu\text{g}/\text{m}^3$ (IQR) increase in PM <sub>2.5</sub> (16.7 $\pm$ 2.3 $\mu\text{g}/\text{m}^3$ )	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)
<b>Kingsley <i>et al.</i> (2016)</b>	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)
<b>Tsamou <i>et al.</i> (2016)</b>	210 mother-child pairs from the ENVIRONAGE cohort (Belgium)	5 $\mu\text{g}/\text{m}^3$ increase in PM <sub>2.5</sub> (16.38 $\pm$ 5.29 $\mu\text{g}/\text{m}^3$ for the first trimester of pregnancy and 16.74 $\pm$ 5.82 $\mu\text{g}/\text{m}^3$ 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.
<b>Saenen <i>et al.</i> (2017)</b>	361 samples from the ENVIRONAGE birth cohort (Belgium)	7.5 $\mu\text{g}/\text{m}^3$ (IQR) increase in PM <sub>2.5</sub> (15.5 $\pm$ 4.9 $\mu\text{g}/\text{m}^3$ for the second trimester)	A 1.4% decrease in <i>LEP</i> promoter methylation for the second trimester of pregnancy (95% CI: -2.7 to -0.2%; p = 0.02)

458

459 Abbreviations: CI, Confidence interval; IQR, Interquartile range; *LEP*, Leptin; miR, Micro RNA; mtDNA, Mitochondrial DNA; PM<sub>2.5</sub>, Particulate matter with a diameter smaller than 2.5  $\mu\text{m}$ .

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

468 Recently, attention has been drawn to the methylation pattern of a specific gene in the  
469 placenta, namely the promoter region of the leptin (*LEP*) gene. Leptin is an important hormone during  
470 pregnancy, since it plays a crucial role in fetal growth and development through its function in energy  
471 metabolism (Walsh *et al.* 2014). An interquartile range increment (IQR) of PM<sub>2.5</sub> exposure (7.5 µg/m<sup>3</sup>)  
472 was associated with a 1.4% decrease in placental methylation of the *LEP* promoter region (95 % CI: -  
473 2.7 to -0.2%; p = 0.02) (Saenen *et al.* 2017). In previous research, a decrease in *LEP* methylation has  
474 been associated with gestational syndromes such as pre-eclampsia (Hogg *et al.* 2013) and impaired  
475 glucose tolerance (Bouchard *et al.* 2010). The intricate connection between *LEP* methylation, PM  
476 exposure, and disease phenotype should be explored more in depth by studying potential ailments in  
477 childhood that may arise from these placental changes.

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

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

488 A type of epigenetic mark that has not yet been investigated to a great extent in the context  
489 of prenatal air pollution exposure is microRNA (miRNA) (Vrijens *et al.* 2015). MiRNAs are endogenous,  
490 single-stranded, short non-coding RNA sequences (approximately 22 nucleotides) that regulate gene  
491 expression at the post-transcriptional level. Different cell types have both common and unique miRNA  
492 expression patterns, which can be influenced by developmental and pathologic states. The human  
493 placenta expresses a distinct subset of miRNAs, but although the functions of these placental  
494 epigenetic marks are largely unknown, recent research has revealed a functional role for miRNAs in  
495 placental biology (Gu *et al.* 2013). The presence of placental miRNAs in the maternal circulation is  
496 interesting as it could lead to the discovery of biomarkers of placental dysfunction or pregnancy-  
497 related disease (Miura *et al.* 2016). Only one study has described changes in placental miRNA  
498 expression in association with prenatal air pollution exposure. A relative decrease in the placental  
499 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 -  
500 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  
501 an increase of  $5 \mu\text{g}/\text{m}^3$   $\text{PM}_{2.5}$  during the second trimester of pregnancy, whereas a positive association  
502 was described between first trimester  $\text{PM}_{2.5}$  air pollution exposure and the expression of placental  
503 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%;  $p =$   
504  $0.015$ ) (Tsamou *et al.* 2016). A common target of these miRNAs is the tumor suppressor phosphatase  
505 and tensin homolog (*PTEN*) which also showed an altered expression in association with  $\text{PM}_{2.5}$   
506 exposure (+59.6% per  $5 \mu\text{g}/\text{m}^3$  increment, 95% CI: 26.9 to 100.7%;  $p < 0.0001$ ) and, as expected, an  
507 inverse correlation with the levels of these miRNAs, since increasing levels of miRNAs are known to  
508 block the expression of their associated targets (Tsamou *et al.* 2016).

#### 509 **4.2.3. Transcriptomics (Table 4)**

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

512 tissue aliquots with a sufficiently high RNA quality for whole transcriptome analyses, since RNA starts  
513 degrading quickly after sampling (Gallego Romero *et al.* 2014). However, various reviews have  
514 discussed the transcriptome of the placenta in the context of negative birth outcomes (Cox *et al.* 2015;  
515 Eidem *et al.* 2015). Recent research has shown that the placenta contains several distinct gene  
516 expression patterns compared with other tissues in the human body, for example when the number  
517 of splice variants and the expression levels of regulators involved in splicing are concerned (Kim *et al.*  
518 2012). Quantitative PCR, micro-array analysis, and RNA sequencing have proven to be indispensable  
519 for the analysis of exposure effects on transcriptomic alterations, potentially leading to perturbation  
520 of developmental and biological mechanisms. Apart from their work in the genomic field, Whyatt *et*  
521 *al.* (1995, 1998) also investigated the effects of prenatal exposure to air pollution on the placental  
522 expression of *CYP1A1*. However, in both studies significant results concerning this gene could not be  
523 established, which was in accordance with the absence of an association in the genomic field of their  
524 research on *CYP1A1* (see section 4.2.1. Genomics).

525         Saenen *et al.* (2015) investigated the placental expression levels of ten genes in the brain-  
526 derived neurotrophic factor (*BDNF*) pathway in connection with PM air pollution. A 5  $\mu\text{g}/\text{m}^3$  increase  
527 in residential  $\text{PM}_{2.5}$  exposure of the mother during the first trimester of pregnancy was associated with  
528 a 15.9% decrease in expression of placental *BDNF* (95% CI: -28.7 to -3.2%,  $p = 0.015$ ), and with a 24.3%  
529 decrease in *synapsin 1* (*SYN1*) expression (95% CI: -42.8 to -5.8%,  $p = 0.011$ ) which is affected by the  
530 actions of *BDNF*. Proper functioning of this pathway in the placenta is crucial for normal fetal  
531 development, since altered *BDNF* expression in this tissue has been associated with negative birth  
532 outcomes such as fetal growth restriction (Mayeur *et al.* 2010).

533 **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 $\pm$ standard deviation if available)	Effect on placental -omics marker
<b>Whyatt et al. (1995)</b>	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 $\mu\text{g}/\text{m}^3$ $\text{PM}_{10}$ in highly polluted area (range, 23.4-154.2 $\mu\text{g}/\text{m}^3$ ), no data available for Limanowa	No significant difference in <i>CYP1A1</i> mRNA levels between low and high polluted area ( $r = -0.4$ ; $p = 0.14$ ).
<b>Whyatt et al. (1998)</b>	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 $\mu\text{g}/\text{m}^3$ of ambient respirable particles in least exposed group and 78 $\mu\text{g}/\text{m}^3$ in the most exposed group, in the year prior to delivery (particle size not defined)	PAH-adduct levels not significantly associated with <i>CYP1A1</i> mRNA ( $r = -0.10$ ; $p = 0.2$ )
<b>Saenen et al. (2015)</b>	90 randomly selected mother-child pairs from the ENVIRONAGE birth cohort (Belgium)	5 $\mu\text{g}/\text{m}^3$ increase in $\text{PM}_{2.5}$ (15.4 $\pm$ 5.4 $\mu\text{g}/\text{m}^3$ for the first trimester, 17.6 $\pm$ 7.0 $\mu\text{g}/\text{m}^3$ for the second trimester and 18.7 $\pm$ 6.0 $\mu\text{g}/\text{m}^3$ for the third trimester of pregnancy)	15.9% decrease in placental <i>BDNF</i> expression (95% CI: -28.7 to -3.2%; $p = 0.015$ ) and a 24.3% lower expression of <i>SYN1</i> (95% CI: -42.8 to -5.8%, $p = 0.011$ )

534

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

#### 537 **4.2.4. Proteomics (Table 5)**

538 Although proteins are the end products of the transcription of genomic sequences, there is no  
539 linear relationship between a genome and its resulting proteome because of alternative splicing and  
540 the production of non-functional proteins (Pandey and Mann 2000). Therefore, it is essential that  
541 proteomics signatures are studied as a separate -omics field which can complement the finding in other  
542 -omics categories (Pandey and Mann 2000). Proteomic characteristics have been investigated in  
543 placental tissue in association with maternal tobacco smoking habits (Machaalani *et al.* 2014) and  
544 other exposures to toxicants during pregnancy. Considerable focus has been put on proteins that play  
545 an essential role in the detoxification system of the cells. The activity of placental aryl hydrocarbon  
546 hydroxylase (AHH), which is the most important metabolizer of PAHs, was significantly higher in  
547 placentas obtained from mothers who lived in an environment exposed to urban air pollution  
548 compared with the control group (Hincal 1986). Two other cellular detoxification indicators,  
549 glutathione S-transferase (GST) and 7-ethoxycoumarin O-deethylase (ECOD), showed opposite  
550 associations with increasing air pollution levels: ECOD activity significantly increased with increasing  
551 ambient air pollution (related to industry and traffic-exhaust), while the GST activity decreased under  
552 the same conditions (Obolenskaya *et al.* 2010). Work-related exposure to another source of PAH air  
553 pollution during pregnancy, such as in the “maquiladoras” at the US-Mexican border, had no effect on  
554 the placental GST level or activity (Dodd-Butera *et al.* 2016). Detoxification processes are important  
555 for normal cellular functioning, but the maintenance of the delicate redox balance of the cell is a crucial  
556 factor as well. In controlling oxidative stress, metallothionein (MT) is an important protein for fixation  
557 and transport of metals. In a study of Sorkun *et al.* (2007), a significant increase in the amount of  
558 placental MT was observed in regions with higher levels of air pollution exposure. Another area of  
559 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 $\pm$ standard deviation if available)	Effect on placental -omics marker
<b>Hincal (1986)</b>	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 ( $p < 0.001$ )
<b>Kedryna et al. (2004)</b>	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 ( $p < 0.001$ )
<b>Sorkun et al. (2007)</b>	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 ( $p = 0.013$ )
<b>Obolenskaya et al. (2010)</b>	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
<b>Saenen et al. (2016)</b>	330 mother-child pairs from the ENVIRONAGE birth cohort (Belgium)	3.5 $\mu\text{g}/\text{m}^3$ (IQR) increase in $\text{PM}_{2.5}$ ( $16.1 \pm 2.4 \mu\text{g}/\text{m}^3$ for whole pregnancy) and 0.36 $\mu\text{g}/\text{m}^3$ (IQR) increase in BC ( $0.97 \pm 0.28 \mu\text{g}/\text{m}^3$ for entire pregnancy)	35.0% increase in 3-NTP levels for increased $\text{PM}_{2.5}$ 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
<b>Dodd-Butera et al. (2016)</b>	$n = 54$ 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

561

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;  $\text{PM}_{2.5}$ , Particulate matter with a diameter smaller  
 564 than 2.5  $\mu\text{m}$ .

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

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

#### 583 **4.2.5. Metabolomics**

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

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

#### 597 4.3. Triple relationship between exposure to ambient air pollution, placental biomarkers 598 and disease development

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

614 **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
<b>Hincal F. (1986)</b>	Urban air pollution	Placental AHH activity	Low birth weight and shorter birth length
<b>Sram et al. (2006)</b>	PM <sub>2.5</sub> , PM <sub>10</sub>	DNA adducts and <i>GSTM1</i> , <i>GSTP1</i> , <i>GSTT1</i> , <i>CYP1A1*2A</i> and <i>CYP1A1*2C</i> genotypes	Low birth weight and prematurity
<b>Rossner et al. (2011)</b>	PAHs, PM <sub>2.5</sub>	SNP analysis for 95 genes and measurement of 8-oxodG adducts	Low birth weight and intrauterine growth restriction
<b>Ghosh et al. (2013)</b>	PAH, PM <sub>2.5</sub>	Six SNPs ( <i>GSTM1</i> , <i>GSTP1</i> , <i>GSTT1</i> , <i>CYP1A1 MspI</i> , <i>EPHX1</i> exon 3 and 4) and one <i>EPHX1</i> diplotype	Acute bronchitis in early childhood
<b>Clemente et al. (2016)</b>	NO <sub>2</sub>	mtDNA content	Low birth weight
<b>Kingsley et al. (2016)</b>	Residential proximity to a major road	DNA methylation (LINE and AluYb8 elements)	Low birth weight and small for gestational age

615

616 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-  
617 transferase M1; *GSTP1*, Glutathione S-transferase P1; *GSTT1*, Glutathione S-transferase T1; His, Histidine; *LEP*, Leptin; miR, MicroRNA; mtDNA, Mitochondrial DNA; *MspI*, Substitution of  
618 isoleucine to valine in the 3' non-coding region of *CYP1A1*, NO<sub>2</sub>, Nitrogen dioxide; PAH, Polycyclic aromatic hydrocarbon; PM<sub>2.5</sub>, Particulate matter with a diameter smaller than 2.5 µm; PM<sub>10</sub>,  
619 Particulate matter with a diameter smaller than 10 µm; SNP, Single nucleotide polymorphism.

620 A significant relationship was identified between the development of childhood bronchitis and the  
621 presence of a low activity *EPHX1* polymorphism in the placenta with increased exposure to PAH and  
622 PM<sub>2.5</sub>. These authors were thereby the first to identify a link between prenatal exposure to air  
623 pollution, a placental -omics marker and disease development.

624 Several studies have focused on placental -omics signatures and adverse birth outcomes such  
625 as reduced birth weight, intrauterine growth restriction, small for gestational age, and prematurity. In  
626 earlier studies, no significant associations were found between ambient air pollution exposure, birth  
627 weight and either the levels of DNA adducts in the placenta (Sram *et al.* 2006) or the activity of aryl  
628 hydrocarbon hydroxylase in placenta (AHH) (Hincal 1986), although a link was shown between air  
629 pollution exposure and AHH activity (see proteomics section). Three more recent studies found  
630 significant negative correlations between birth weight and the levels of NO<sub>2</sub> (Clemente *et al.* 2016),  
631 PM<sub>2.5</sub> and PAHs (Rossner *et al.* 2011), and the residential proximity to a major road (Kingsley *et al.*  
632 2016). However, Rossner *et al.* (2011) concluded that the levels of 8-oxo-deoxyguanosine (8-oxodG)  
633 DNA adducts, a marker of direct oxidative DNA damage, measured in placental tissue were only  
634 correlated with PM<sub>2.5</sub> levels but not with birth weight. The same conclusion was reached for the  
635 association between residential distance to a major road and LINE element methylation: LINE  
636 methylation levels were only negatively associated with a distance to a major road, but no link with  
637 birth weight could be identified (Kingsley *et al.* 2016). The only mediation analysis to investigate the  
638 triple association between air pollution exposure, placental -omics and disease development was  
639 performed with data of the INMA birth cohort (Clemente *et al.* 2016). This analysis showed that 10%  
640 of the association between a 10 µg/m<sup>3</sup> increase in NO<sub>2</sub> exposure during pregnancy and reduced birth  
641 weight could be mediated by a decrease in mitochondrial DNA levels. The results of these three studies  
642 could suggest that prenatal exposure to air pollution might exert its effects on birth outcomes by  
643 altering more subtle regulations such as those of the placental energy system and not by direct damage  
644 to the placental DNA. However, since a broad array of possible molecular factors could be responsible  
645 for the link between *in utero* air pollution exposure and an effect on disease development later in life,

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

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

658

## 659 **5. CONCLUSIONS AND FUTURE DIRECTIONS**

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

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

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

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

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

## 705 **6. DECLARATIONS**

### 706 **6.1. COMPETING FINANCIAL INTERESTS DECLARATION**

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

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719 **6.4. AUTHORS' CONTRIBUTIONS**

720 Selection criteria for the in- or exclusion of articles were determined by LJJ, NDS, and TSN. The  
721 literature search was performed by LJJ and NDS, and TSN was consulted if any discrepancies  
722 remained between these two researchers. LJJ wrote the first draft of the systematic review, with  
723 contributions from NDS (suggestions on section "4.1. Placental tissue in epidemiological research:  
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726 finalization of the manuscript. All authors read and discussed the final version of the manuscript  
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728

729

730 **7. REFERENCES**

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