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Alterations in the placental proteome in association with the presence of black carbon particles: a discovery study

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ABSTRACT

Background: Exposure to ambient air pollution is known to cause direct and indirect molecular expression changes in the placenta, on the DNA, mRNA, and protein levels. Ambient black carbon (BC) particles can be found in the human placenta already very early in gestation. However, the effect of *in utero* BC exposure on the entire placental proteome has never been studied to date.

Objectives: We explored whether placental proteome differs between mothers exposed to either high or low BC levels throughout the entire pregnancy.

Methods: We used placental tissue samples from the ENVIR*ON*AGE birth cohort, of 20 non-smoking, maternal- and neonate characteristic-matched women exposed to high (n=10) or low (n=10) levels of ambient BC throughout pregnancy. We modeled prenatal BC exposure levels based on the mother's home address and measured BC levels in the fetal side of the placenta. The placental proteome was analyzed by nano-liquid chromatography Q-TOF mass spectrometry. PEAKS software was used for protein identification and label-free quantification. Protein-protein interaction and functional pathway enrichment analyses were performed with the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) software.

Results: The accumulation of BC particles in placenta was 2.19 times higher in the high versus low exposure group (20943.4 vs 9542.7 particles/mm³; p=0.007). Thirteen proteins showed a ≥ 2-fold expression difference between the two exposure groups, all overexpressed in the placentas of women prenatally exposed to high BC levels. Three protein-protein interactions were enriched within this group, namely between TIMP3 and COL4A2, SERPINE2 and COL4A2, and SERPINE2 and GP1BB. Functional pathway enrichment analysis put forward pathways involved in extracellular matrix-receptor interaction, fibrin clot formation, and sodium ion transport regulation.

Discussion: Prenatal BC exposure affects the placental proteome. Future research should focus on the potential consequences of these alterations on placental functioning, and health and disease during early childhood development.

Key words: black carbon particles, placental proteome, mass spectrometry, prenatal air pollution exposure, exposome

1. INTRODUCTION

The effect of ambient combustion-derived air pollution exposure on placental protein expression has not been studied to a great extent [1]. Only several pre-selected targets have been linked with exposure to air pollution during pregnancy, with the largest focus on proteins with a role in the cellular detoxification system [2-4]. The association between combustion-derived air pollution exposure and the protein expression of one specific marker, 3-nitrotyrosine, has been previously studied [5]. However, environmental proteome studies can provide important insights into the affected mechanisms at the molecular level [6]. Black carbon (BC) particles have been detected in the fetal side of the human placenta, as early as 12 weeks of gestation [7]. The quantity of particles in placental tissue was strongly and positively associated with the ambient residential modelled BC levels to which the mother had been exposed throughout pregnancy.

In utero BC exposure has been associated with increased mitochondrial methylation levels [8], an elevated Alu mutation rate (a marker of general DNA mutation) and an increased promoter methylation in APEX1 and ERCC4 [9]. In addition, prenatal air pollution has been associated with shorter telomere length, a marker of aging, at birth [10]. In the placenta specifically, BC exposure has been shown to cause multiple adverse effects, such as oxidative stress, inflammatory responses, changes in gene expression, nitrosative stress, and altered iodine uptake [5, 11-13].

However, to our knowledge, the association between *in utero* exposure to ambient air pollution such as BC and the induced placental changes on a proteome-wide level has never been examined. The effect of BC exposure on the proteome has been studied in several (non-placental) cell lines and animal models. In the human lung epithelial A549 cells, BC upregulated 9 specific proteins compared to for example TiO_2 exposure, related to cell proliferation and apoptosis [14]. Label-free nano LC-MS/MS analyses of bronchoalveolar lavage fluid in BC-exposed mice revealed a pattern of specific up- and down-regulated proteins related to inflammation and surfactant production [15].

Studying the effects of the BC exposure on the protein level is a crucial part of unraveling associations between combustion-derived air pollution exposure and molecular changes in the placenta. Together with results on other expression levels, it will provide a broad understanding of possible effects of environmental stressors on placental development and functioning, and consequently, on fetal development and human health. Therefore, we examined the association between exposure to ambient BC air pollution during pregnancy and the placental proteome at birth. For this purpose, we compared placental tissue samples of the ENVIR*ON*AGE prospective birth cohort of women exposed to either high or low levels of BC and analyzed the protein composition by means of label-free nano LC-MS/MS analysis.

2. METHODS

2.1 Recruitment and eligibility

This study population is part of the ongoing prospective ENVIR*ON*AGE (ENVIRonmental influence *ON* AGEing in early life) birth cohort [16]. The study was conducted according to the guidelines

described in the Declaration of Helsinki, and the study protocol was approved by the ethics committees of the East-Limburg Hospital (Genk, Belgium) and Hasselt University (Diepenbeek, Belgium). The twenty mothers of whom the samples were used in this project were recruited at the maternity ward of the East-Limburg Hospital upon delivery between 12 am on Fridays and 7 am the subsequent Mondays, between the 18th of February 2012 and the 6th of October 2016. All participants signed an informed consent document before giving birth.

Women were eligible for participation if they carried a singleton pregnancy and were able to fill in a Dutch questionnaire. This questionnaire, filled in after delivery, provided us details on both clinical information, such as the mothers' age and lifestyle information, such as their educational level, smoking habits, alcohol consumption, and residential address(es) throughout the entire pregnancy. The neonate's date of birth, gestational age, sex, birth weight, and birth length were obtained from their medical files, as well as the maternal parity and obstetric complications such as gestational diabetes, hypertension, and preeclampsia. Maternal education, as a proxy for socio-economic status, was categorized as low (no diploma or primary school), middle (high school diploma), and high (college or university degree) according to the ISCED classification. Since (e-)cigarette smoke is a source of BC [17], we selected maternal smoking during pregnancy as an exclusion criterium for this study. Moreover, all births included are natural deliveries, c-sections were excluded. This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines.

2.2 Exposure assessment

In our study, we assessed both ambient BC exposure levels and BC particle load at the fetal side of the placenta. Exposure to ambient BC during pregnancy was determined by the mothers' residential address for all ENVIR*ON*AGE participants, by a high resolution spatial-temporal interpolation method [18]. This method combines a dispersion model with air pollution exposure data provided by official fixed monitoring stations in Flanders, and land cover data obtained from satellite images of the CORINE land cover dataset [19, 20]. This model chain provides interpolated air pollution values from the Belgian telemetric air quality networks on a dense, irregular high-resolution receptor point grid from both point sources (such as industrial sites), and line sources (such as highways or major roads). The overall model performance was assessed by leave-one-out cross-validation, including 16 monitoring stations for BC. Validation statistics determined a spatiotemporal explained variance of more than 74%. Moreover, the accuracy of the exposure models was recently demonstrated by the association between the urinary load of nano-sized BC particles in children and their residential BC levels [21].

To determine the duration of the entire pregnancy period, the conception date was estimated by the first day of the mother's last menstrual period and ultrasound imaging data [22]. Address changes during pregnancy for two participants were taken into account while modelling the residential ambient BC exposure. High residential ambient BC exposure was defined as having both an entire pregnancy and third trimester residential BC exposure ≥ 75th percentile (1.70 μg/m³ and 2.42 μg/m³, respectively), and a residential proximity to a major road ≤ 500 m. Low residential ambient BC exposure was defined as having both an entire pregnancy and third trimester residential BC exposure ≤ 25th percentile (0.96 μg/m³ and 0.63 μg/m³, respectively), and a

residential proximity to a major road > 500 m [7]. Among the 960 eligible participants of the ENVIR*ON*AGE birth cohort recruited within the period of this study, 91 were classified as high exposed and 69 as low exposed during pregnancy. Following the exclusion of mothers who smoked, 56 participants remained in the high exposure group and 44 in the low exposure group. We then randomly selected 10 participants from each of the two exposure groups. The two groups were matched based on neonate- and maternal characteristics.

Next, the placental tissue BC load was determined for these 20 samples by means of non-incandescence-related white-light generation of the BC particles under femtosecond illumination, as described previously [7, 21]. Placentas were collected at the hospital within 10 minutes after birth. At approximately 4 cm from the umbilical cord, biopsies were taken at four standardized sites at the fetal side of the placenta [7]. The BC load was measured in three of these four placental biopsy regions, within five different sections taken in the middle of the biopsy (n = 15 images). The samples were fixed in formaldehyde for at least 24 hours and embedded in paraffin. Tissue sections of 4 µm were obtained with a microtome (Leica Microsystems). To avoid ambient BC particulate contamination, sections were made in a clean room with filtered air (Genano 310/OY) using particle-free instruments and sample holders.

Images of the placental fractions were obtained with a Zeiss LSM 510 confocal laser scanner (Carl Zeiss) containing a two-photon femtosecond pulsed laser (MaiTai DeepSee, Spectra-Physics). The scanner was pre-set to a central wavelength of 810 nm, on average 5 or 10 mW radiant power, with a 10×/0.3 objective. White-light emission produced by the BC particles was obtained with two filters, between 400–410 nm and 450–650 nm, to image both two-photon excited autofluorescence of the placental cells and second harmonic generation from collagen type I, respectively. A tile scan of 100 images was acquired within a field view of 9000 × 9000 μm^2 with a pixel size of 0.694 µm and a pixel dwell time of 2.51 µs. The number of BC particles in the scans of each placental section was determined with an automated and customized Matlab program (Matlab 2010, Mathworks). The carbonaceous nature and intra-tissue localization of the identified black carbon particles was confirmed using rigorous validation experiments, as described by previous studies [7, 23].

2.3 Placental tissue sampling, protein extraction and protein digestion

Placentas were collected and deep-frozen within 10 min after childbirth. Tissue samples were taken according to a standardized method at four fixed sites on the fetal side of the placenta, four cm from the center of the umbilical cord. The position of the sites was determined clockwise by the largest placental vein. Samples were taken 1.0 cm to 1.5 cm beneath the chorio-amniotic membrane and were snap-frozen immediately and kept at -80°C until protein extraction.

Proteins were extracted from a pooled sample of all four quadrants of each placenta, with a final weight of 40 mg. The below-described protocol for sample treatment, protein extraction and peptide digestion was performed as optimized previously [24]. All pooled samples were thoroughly washed with phosphate-buffered saline (PBS) before freezing and a second time after thawing, and were subsequently put in 300 µL extraction buffer, consisting of 2% CHAPS, 2% DTT, 2 M thiourea, and 7 M urea. Tissue samples were disrupted mechanically with an Ultra Turrax T8 mixer (IKA) three times during 10 sec. Subsequently, the lysates were incubated on a shaking plate for 30 min

at 1400 rpm and 15°C and subsequently centrifuged for 5 min at 16,000 g. Protein concentration was determined in the resulting supernatant with the Pierce BCA Protein Assay Kit (Thermo Scientific). Extracts were kept at -80°C until protein digestion.

Protein extracts were digested using modified filter-aided sample preparation (FASP) method as described by Wiśniewski *et al.* [25] and Distler *et al*. [26]. All centrifugation steps were performed at 11,000 g. In short, 10 µg of protein was added onto the filter, followed by 200 µL of 8 M urea acetate (UA). After 15 min of centrifugation, the protein mixtures were reduced with 8 mM dithiothreitol (DTT) while incubating at 56°C for 15 min. Next, the samples were alkylated with 50 mM iodoacetamide (IAA) during a 20 min incubation at room temperature, and following centrifugation and a washing step with UA, excess IAA was removed by a second incubation with DTT. After three washing steps, one with UA and two with 50 mM NH_4HCO_3, proteins were digested with trypsin (1:50 enzyme to protein) for 5 hours at 300 rpm. Following a final centrifugation of 10 min at 13,000 g, the digestion was stopped by adding 2% trifluoroacetic acid (TFA; Biosolve) and the resulting peptide mixture was analyzed with nano LC-MS/MS.

2.4 Nano LC-MS/MS

The digest was analyzed using nano-LC-ESI-MS/MS timsTOF Pro (Bruker) coupled with a UHPLC nanoElute (Bruker). For each digest, two injections were performed, resulting in two technical replicates per biological sample. Peptides were separated by nanoUHPLC (nanoElute, Bruker) on a C18 column (75 µm ID, 25 cm) with integrated CaptiveSpray insert (Aurora, Ionopticks) at a flow rate of 400 nl/min and 50°C. LC mobile phase A contained water with 0.1% formic acid (v/v) and phase B consisted of ACN with 0.1% formic acid (v/v). Samples were loaded directly onto the analytical column at a constant pressure of 800 bar. One microliter of the peptide mixture was injected, and the organic content of the mobile phase was increased linearly from 2% to 15% phase B in 60 min, from 15% to 25% phase B in the next 30 min, from 25% to 37% phase B in the subsequent 10 min and from 37% to 95% phase B in the final 5 min. Data acquisition on the timsTOF Pro was performed using Hystar 5.0 and otof-Control 6.0., using 160 ms TIMS accumulation time, and a mobility (1/K0) range from 0.7 to 1.4 Vs/cm². Mass-spectrometric analysis was carried out using the parallel accumulation serial fragmentation (PASEF) acquisition method [27]. One MS spectrum was followed by six PASEF MSMS spectra per total cycle of 1.16 s.Data analysis was performed using PEAKS Studio X+ with ion mobility module and Q module for label-free quantification (Bioinformatics Solutions Inc.) [28]. Protein identifications were conducted using the PEAKS search engine with 15 ppm as parent mass error tolerance and 0.05 Da as fragment mass error tolerance. Carbamidomethylation was allowed as fixed modification, oxidation of methionine and acetylation (N-term) as variable modification. Enzyme specificity was set to trypsin, and the maximum number of missed cleavages per peptide was set at one. The peak lists were searched against the Homo Sapiens and isoforms protein database from UNIREF 100 (173190 sequences). Peptide spectrum matches and protein identifications were normalized to less than 1.0% false discovery rate (FDR). Label-free quantitation (LFQ) is based on a determination of the area under the curve and calculates the expectation-maximization algorithm on the extracted ion chromatograms of the three most abundant unique peptides of a protein [29]. For protein quantification, the mass error was set to 20 ppm, ion mobility tolerance to 0.08 1/k0, peptide

quality score to ≥ 4 and the protein significance score threshold was set to 20. The significance score was calculated as the $-10\log_{10}$ of a p-value of 0.01. The software used an ANOVA test to determine statistical significance. Modified peptides were excluded and only proteins with at least two identified peptides were used for quantification. Total ion current was used to calculate the normalization factors.

To ensure the quality of the proteome analysis, automatic calibration of ion mobility was performed for each analysis. Moreover, the samples were randomly injected and the technical replicates were injected during a second series of analyses. To check run-to-run reproducibility of the technical replicates, the abundance correlation for the detected protein was checked in PEAKS using the Pearson correlation score, which indicates the reproducibility of the experiment based on a selected pair of samples.

2.5 Western Blot

Protein expression verification of the LC-MS/MS results was performed for SERPINE2 and TIMP3. We used Color Prestained Protein Standard, Broad Range (New England BioLabs) as protein standard. Twenty micrograms of each extract were loaded onto a 4-20% Mini-PROTEAN® TGX™ Precast Protein gel (10 well - 30µl, ref #4561093, Bio-Rad), which were run for 40 min at 200 V. Subsequently, proteins were blotted with the Trans-Blot® Turbo™ RTA Mini LF PVDF Transfer Kit (Bio-Rad), according to the manufacturers' instructions. We used the fluorescent immunostaining of GAPDH as loading control. Primary antibodies used for this experiment were mouse anti-SERPINE2 (#MA5-25936, Thermo Fisher Scientific) and rabbit anti-TIMP3 (CS #5673, Cell Signaling Technology) and rabbit anti-GAPDH (#ab128915, Abcam), and IRDye 800CW-conjugated goat anti-rabbit (#926-32211, LI-COR Biotechnology) and IRDye 680RD-conjugated goat anti-mouse (#926-68070, LI-COR Biotechnology) were used as secondary antibodies. Both primary antibodies were diluted in Odyssey Blocking Buffer (PBS) (LI-COR Biotechnology) and 0.1% Tween (1000x for SERPINE2 and TIMP3 and 10,000x for GAPDH) and incubated onto the membrane, overnight at 4°C for SERPINE2 and TIMP3, and 30 min at room temperature (RT) for anti-GAPDH. The secondary antibody was diluted 10,000 times in the same medium and incubated at RT for one hour. Imaging and relative quantitative fluorescence analyses were performed with the Odyssey Classic Infrared Imaging System (LI-COR Biotechnology). Normalized expression values were obtained by calculating the ratio of the target protein and GAPDH.

2.6 Statistical analysis

We used a two-sided Student's t-test to determine potential differences in participant characteristics between both exposure groups for continuous variables, and the Fisher's Exact test for categorical variables. We performed protein quantification and determined differential expression values between the two groups by means of PEAKS software. To determine differentially expressed proteins, the PEAKS algorithm used a two-way ANOVA, with a set significance of 20 (corresponding to a p-value of ≤ 0.01) and a fold change of ≥ 2 (and an additional, exploratory analysis with a fold change of ≥ 1.5) and ≥ 2 peptides per protein used for quantification. The fold change refers to the expressed protein ratios between the two exposure groups. Finally, we set in the PEAKS software that a peptide had to be detected in at least 60% of the analyzed samples per

group. For all protein groups that passed the former quantification filters, the software used Benjamin–Hochberg multiple testing correction to adjust the p-value for the FDR calculation. Only proteins with significant scores passing the adjusted FDR were provided in the final listing.

2.7 Protein-protein interaction analysis

For protein-protein interaction analysis, we used the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) online software (version 11.0). This analysis gives the number of interactions between the entered proteins and compared these against the number of interactions that would be expected from a random set of proteins. Furthermore, STRING determines the functional pathways and systems that are enriched in a set of proteins, using a combination of several online classification systems such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) [30] and Gene Ontology (GO) [31] databases to determine and describe protein-protein interactions in terms of enriched functional associations [32, 33]. STRING pathway enrichment calculations apply Benjamini-Hochberg multiple testing correction, within each separate functional classification framework (such as GO and KEGG) [33].

3. RESULTS

3.1 Population and exposure characteristics

The mothers in the low and high exposure group had similar characteristics, as summarized in **Table 1**. On average (standard deviation), they gave birth at the age of 29.9 (4.2) years, with a parity of 1.8 (1.1). The education level of the mothers was high (90.0%), and two mothers of the high exposed group drank occasionally a glass of alcohol during pregnancy. Moreover, two mothers of the low exposed group suffered from gestational diabetes, while both groups contain one participant with hypertension. None of the participants had a preeclamptic pregnancy. The average ambient BC exposure level in the low exposure group was 0.82 (0.09) μg/m³, and 1.94 (0.24) μg/m³ in the high exposure group. Average placental BC load reflected the ambient levels, with a 2-fold higher average carbon load in the high exposure group (20943.4 (9642.3) particles/mm³) compared to the low exposure group (9542.7 (6645.7) particles/mm³). The neonates (50.0% girls) had an average gestational age of 39.8 (2.0) weeks, weighed 3395.0 (438.7) g and had a length of 49.9 (2.0) cm. None of these variables showed a significant difference between the high and the low exposure group. **Supplemental Table S1** provides a comparison of the characteristics between the 20 selected samples and all the recruited mother-newborn pairs form the cohort between the 18th of February 2012 and the 6th of October 2016 (n=1050). However, for 90 mother-newborn pairs there was missing information about at least one of the described characteristics, therefore the overview is based on 960 samples. As we selected specifically for non-smoking mothers and specific ambient BC exposure levels, these two parameters were significantly different compared to the total cohort population (p=0.088; p=0.017 respectively). All other parameters did not show a significant difference when applying the 0.05 significance level.

3.2 Protein identification

For each biological sample we analyzed two technical replicates, this resulted in a total of 40 samples used for protein identification and quantification. In these 40 samples combined, 64,373 unique peptides and 8556 unique proteins were identified. Pearson correlations between technical replicates of each sample, and between biological replicates of the same BC exposure group were assessed based on all quantified proteins, as demonstrated in **Supplemental Figure S1** and **Supplemental Figure S2**. These analyses indicated that the correlations for all technical and biological replicates were positive and significant, with $p<0.001$. Moreover, sample clustering showed that the two technical replicates of each of the 20 biological samples were strongly clustered, validating the low technical variability in protein identification (**Figure 1**). It also identified a strong clustering between the samples within the same BC exposure group, which verifies the great similarity in protein profile within the samples of either the high or low BC group.

3.3 Increased protein expression in high black carbon exposure group

Two-way ANOVA statistical analysis identified 13 FDR-significant proteins with a ≥ 2-fold different expression between placentas from the high and low prenatal BC exposure groups (**Table 2**). All proteins showed a higher expression in the placentas of women exposed to high levels of prenatal BC. Protein-protein interaction (PPI) enrichment analysis showed that between the 13 proteins (nodes) there were 3 significant interactions (edges), while none were expected, with a PPI-enrichment p-value of 0.015 (**Figure 2**). Enriched PPIs were identified between metalloproteinase inhibitor 3 (TIMP3) and collagen alpha-2(IV) chain (COL4A2), glia-derived nexin (SERPINE2) and COL4A2, and SERPINE2 and platelet glycoprotein Ib beta chain (GP1BB). Pathway enrichment analysis in STRING put forward the extracellular matrix (ECM)-receptor interaction pathway to be enriched from the KEGG pathway databases, two processes were enriched in the GO databases of Biological Process, namely the regulation of sodium ion transport and intermediate filament organization and two pathways from the Reactome Pathways database were determined to be enriched, related to fibrin clot formation and hemostasis (**Table 3**).

For a more exploratory view on the data, we performed a second functional enrichment analysis in STRING, taking into account proteins that showed at least a 1.5-fold difference in expression between the two exposure groups. In total, 59 proteins with a ≥ 1.5-fold change were FDR-significantly different between the high and low exposure groups, with 41 proteins being overexpressed in the high exposure group, and 18 proteins in the low exposure group (**Figure 2 and Supplemental Table S2**). In the high exposure group, apart from the ECM-receptor interaction pathway to be enriched from the KEGG database, seven additional pathways were enriched (**Supplemental Table S3**). In the low exposure group, no KEGG or GO pathways were enriched within the 18 proteins, although there were four significant protein-protein interactions within this group, between CYTC and CDA, GCSH and ALDA4H1, GLRX and IBA57, and between PHF5A and PFDN5.

3.4 Western Blot verification of SERPINE2 and TIMP3 protein expression

Relative protein expression results of SERPINE2 and TIMP3 show a strong correlation (95% confidence intervals) with their relative expression results on the LC-MS/MS level, with r = 0.75 (0.79, 0.98); p<0.001 for SERPINE2 and r=0.70 (0.74, 0.99); p<0.001 for TIMP3 (**Supplemental Figure S3 and S4**). A one-sided student t-test shows a significant difference in Western Blot protein expression between the low and high BC exposure group for both SERPINE2 (p = 0.035) and TIMP3 (p = 0.036).

4. DISCUSSION

In this study, we examined the association between prenatal exposure to BC air pollution and the placental proteome. We found that 13 proteins were ≥ 2-fold upregulated in the placentas of mothers who were highly exposed to BC, compared to those of the low exposure group. Protein-protein interaction analysis showed that GP1BB, SERPINE2, COL4A2 and TIMP3 had a stronger interaction than expected by chance. Through pathway enrichment analysis, we identified five pathways linked to the ≥ 2-fold upregulated proteins, related to sodium ion transport, the formation of fibrin clots, hemostasis, intermediate filament organization and the ECM-receptor interaction pathway, indicating the disruption of these cellular processes in association with elevated levels of prenatal ambient BC exposure.

Besides the 13 identified proteins, 59 proteins were differentially expressed when using a 1.5- or higher fold change in expression as cut-off. In a study from Wang and colleagues, comparing the placental proteome of normal and preeclampsia pregnancies, preeclampsia was shown to cause differential expression of 171 placental proteins with at least a 1.5-fold change [34], while another study on preeclampsia using 2DE and MALDI-TOF MS identified 20 differentially expressed spots from placental tissue extracts [35]. When studying direct exposure to black carbon (and many other) particles via cigarette smoke, Huuskonen and colleagues showed by means of 2-D gel separation and spot picking that 18% of the protein spots were differentially expressed in mothers who smoked during pregnancy [36]. However, the authors took into account less stringent thresholds: a 1.2-fold difference, compared to a ≥ 2-fold difference in our study.

The thirteen differentially expressed targets from our analyses were identified only within the high BC exposure group. None of these proteins have been associated with BC or other (prenatal) air pollution exposure. However, SERPINE2 has been associated with gestational maternal smoking and childhood pulmonary function in the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) cohort [37]. This prospective study, containing the genotyping records of 1996 children aged 8 years [37], showed that children with *SERPINE2* allele SNP rs729631 had a higher forced vital capacity (FVC) suggesting a protective function against the effects of maternal smoking during pregnancy.

One of the enriched protein-protein interactions detected was between extracellular matrix proteins COL4A2 and TIMP3. Both COL4A2, one of the subunits of collagen type 4 which forms an important structural component of basement membranes, and TIMP3, an inhibitor of matrix metalloproteinases that break down the ECM, are overexpressed in the placental tissue of pre-eclampsia pregnancies [38, 39]. The increased expression of these genes is thought to contribute to the physiological characteristics of this condition: dysregulated COL4A2 would result in shallow trophoblast invasion and an increased production of the COL4A2 product canstatin could contribute to anti-angiogenic effects such as increased apoptosis and endothelial dysfunction [39, 40]. Increased expression of TIMP3 in preeclamptic conditions, potentially regulated by the hypomethylation of the promotor, could result in a lesser degree of trophoblast invasion, eventually resulting in hypoperfusion of the placenta [41]. Apart from this route, TIMP3 also has anti-angiogenic properties by blocking the binding of vascular endothelial growth factor (VEGF) to the VEGF receptor-2, potentially contributing to the adverse placental conditions identified for preeclampsia [42]. Since pre-eclampsia has been associated with prenatal exposure to (BC) ambient air pollution [43, 44], the link between BC air pollution exposure during gestation and TIMP3 in association with the occurrence of preeclampsia should be further investigated.

Pathway enrichment analysis identified the ECM-receptor interaction pathway, which contains 88 protein coding genes, with COL4A2 and GP1BB as overexpressed targets in our analyses. This ECM-receptor interaction system has been identified as being overexpressed in several cancer models, such as pulmonary adenocarcinoma (especially in non-smokers) [45], breast cancer [46] and natural killer/T-cell lymphoma [47]. It is assumed that an overexpression of ECM components results in solid stress in tumors, and contributes to compression of blood vessels, reducing perfusion and consequently accessibility to therapeutics [48, 49]. Moreover, in a $PM_{2.5}$ exposure-induced lung injury model in rats, Zhang and colleagues [50] recently found the ECM-receptor interaction pathway to be upregulated after seven days of $PM_{2.5}$ exposure. Since reduced perfusion in placental tissue is a known characteristic of for example preeclampsia pregnancies [51], we would advise future research to investigate perfusion of the placenta in association with ambient BC air pollution exposure.

A previous study on urinary proteomics in adults shows that the ECM-receptor interaction pathway is strongly related to aging and reported that the urinary peptide COL4A2 is significantly associated with age [52, 53]. Besides the ECM-receptor interaction pathway, the second functional enrichment analysis in STRING reveals seven additional enriched pathways with a ≥1.5 fold change in the high exposure group, five of which overlap with pathways found to be related with aging by Martens *et al.*, namely focal adhesion, AGE-RAGE signaling pathway in diabetic complications, platelet activation, human papillomavirus infection and PI3K-Akt signaling pathway [52]. A review of Johnson *et al*. on human proteomics aging studies also confirms an overlap with the pathways of focal adhesion, PI3K-Akt signaling and hemostasis [54]. Furthermore, SERPINE2 and TIMP3 expression have both been linked with cellular senescence, respectively in human and murine fibroblasts, and in human mesenchymal stem cells [55, 56]. Additionally, the methylation status of

a CpG located in the gene *ITGA2B* has been shown to be one of three CpGs with very high predictive capacity for aging in blood [57]. Seven of the eight enriched pathways with a fold change of ≥1.5 revealed by STRING analysis in our study contain the ITGA2B protein. These findings might indicate an impact of ambient air pollution exposure during pregnancy on placental aging and on the cellular senescence associated with it [58], and will need to be examined in future research.

Together with increased PKP2 and ATP2B4 protein expression, SERPINE2 is also part of the regulation of the sodium ion transport, one of the enriched pathways in this analysis. The effect of prenatal air pollution exposure on placental sodium ion transport has not been studied to date. However, previous research indicates that the activity of multiple nutrient transporters, is decreased in placentas of fetuses with growth restriction, which suggests that changes in nutrient transport may contribute to adverse fetal development [59]. Sodium ion transport is one of the main systems providing active transport of nutrients, also in the placenta. Moreover, it has been shown that activity and protein expression of sodium ion transport within the placental syncytiotrophoblast cells is associated with fetal growth [60]. It is believed that this transport influences the pH homeostasis and cell volume regulation within these placental cells [60]. This might suggest a possible mechanism through which prenatal BC exposure could affect fetal growth and development, which will need to be further validated and examined.

Hemostasis and fibrin clot formation came forward as the two enriched pathways with the lowest FDR (i.e. the lowest chance of these pathways to be discovered by chance) within our enrichment analysis. The effect of prenatal air pollution exposure on placental hemostasis and fibrin clotting has not been studied to date. However, maternal smoking during pregnancy has been found to increase the creation of fibrin clots and disturb fibrin deposition in placental tissue, potentially affecting the transport of oxygen and nutrients towards the fetus [61]. The effects of air pollution on fibrin clotting have been described in the systemic hemostatic process. In a group of 137 non-smokers with both type 1 and type 2 diabetes, an IQR increase of 39.2 $\mu g/m^3$ in PM_{10}, measured 2 hours before patient examination, was associated with a 21.1 sec decrease in the closure time of the Platelet Function Analyzer-100 (PFA-100), which is an indication for increased platelet activation. For long-term exposure, an area increase of 0.25 μm^2 in carbon load of airway macrophages was associated with an increase of 687 leukocytes per microliter of blood [62]. Also for long-term BC exposure, increasing BC levels were associated with an increase in fibrinogen measured in plasma samples from 6814 adults of the Multi-Ethnic Study of Atherosclerosis (MESA) study [63].

STRING analysis of the proteins with a ≥ 1.5-fold difference in expression shows a GO classification of the proteins into several functional groups. In the group of proteins showing a higher expression in the group of mothers prenatally exposed to high levels of BC, we observe a protein functionality that is more directed towards vascular and structural functioning of the placenta. From a vascular perspective, 5 proteins (SERPINE1, FLT1, COL4A2, ECM1, and ITGB3) were involved in

angiogenesis of which 3 proteins (FLT1, ECM1 and ITGB3) have a known function in endothelial proliferation. A potential mechanism for increased expression of these proteins following BC exposure is the activation of hypoxia-related responses. It is known that exposure to air pollution can trigger hypoxic conditions in several tissues, such as the placenta, which in turn can lead to altered development of lung tissue [64]. Placental hypoxia can be considered a normal event during several phases of pregnancy and is required for proper placentation and angiogenesis. However, when decreased oxygen pressure occurs in other developmental stages, this can lead to inadequate fetal cardiovascular and neurological development [65]. SERPINE1 protein levels have been shown to increase under low oxygen levels in placental cells cultures, but also ITGB3, ECM1 and FLT1 expression have been shown to increase under hypoxic conditions [66-69].

Another important group of GO classifications involved cellular interaction. Of the 41 proteins with placental overexpression in highly exposed women, 9 proteins could be linked to the negative regulation of cellular signal transduction. Seven proteins were linked to the (positive or negative) steering of cellular migration and 2 proteins, fibrillin-1 (FBN1) and integrin beta-3 (ITGB3), were associated with cell adhesion mediated by integrin. It is assumed that a disturbance of the balance between contractile structures and extracellular matrix components such as FBN1 and ITGB3 can alter proper functioning of the placenta. For example, in the placentae of preeclampsia pregnancies, there is a significant overexpression of FBN1 [70].

When taking into account the proteins with at least a 2-fold difference in expression, the proteins identified only showed overexpression in the placentas of mothers exposed to high levels of BC exposure during pregnancy. However, when we included proteins with at least a 1.5-fold expression change, we found several proteins with a significantly lower placental expression. In this group of 18 proteins, STRING functional pathway analysis shows that 7 proteins were associated with mitochondria, namely Protein SCO1 homolog (SCO1), 3-ketoacyl-CoA thiolase (ACAA2), Glycine cleavage system H protein (GCSH), Putative transferase CAF17 (IBA57), Enoyl-CoA hydratase domain-containing protein 3 (ECHDC3), Delta-1-pyrroline-5-carboxylate dehydrogenase (ALDH4A1), and SRA stem-loop-interacting RNA-binding protein (SLIRP). The effect of air pollution exposure on mitochondria in the placenta has been studied mostly on the level of the mitochondrial DNA (mtDNA). In the ENVIR*ON*AGE cohort, increased exposure to $PM_{2.5}$ air pollution over the entire pregnancy was associated with a 15.6% decrease in placental mtDNA content and increased mtDNA methylation, which is indicative of decreased DNA expression in mitochondria [8, 71]. The same relation was found for prenatal NO_2 exposure, with a 4.9% decrease in placental mtDNA content for every 10 $\mu g/m^3$ increment in NO_2 exposure during the entire pregnancy period. This study also showed that mtDNA content was associated with birth weight in the Spanish INMA cohort, with mtDNA content as a mediatory effect in this relationship [72].

In subsequent research, the differently expressed proteins identified in this study should be verified both on the protein level, for example by Western Blot, and on different expression levels. Indeed, looking solely at the proteome does not give a complete image of the association between environmental exposure and changes on the molecular level. Furthermore, future investigations should focus on the associations of other fractions of air pollution and other (daily) environmental stressors on the placental proteome to be able to complete the molecular puzzle on the effects of the environment on placental functioning and, consequently, the potential effects on human health.

This study has several strengths and limitations. The strengths are that our study is the first to describe the relationship between prenatal exposure to ambient air pollution and the associated changes in the placental proteome. Secondly, we were able to use very accurate data on the ambient BC exposure of our participants, with the mothers being exposed to relatively low average annual ambient BC concentrations (ranging between 0.63 - 2.42 $\mu g/m^3$) [7]. Finally, measuring the BC particle load of placental samples provides a direct and exact measure of BC exposure during pregnancy. We acknowledge the following limitations. Firstly, the characteristics of the high and low exposure groups are identical or similar. Moreover, we compared the 20 selected samples to the whole ENVIR*ON*AGE birth cohort, which showed similar characteristics. Therefore, the risk of selection bias should be minimal. However, we cannot fully rule out selection bias by unmeasured variables or the possibility that other confounding variables could influence the observed associations. Secondly, we could only examine and compare the placental proteome for 20 individuals, limiting our population sample size. Considering technical replicates and the machine time needed for each sample, current techniques are limited in the number of analyses that can be performed. As techniques progress, research should focus on measuring a larger number of participants in prospective birth cohorts.

In conclusion, BC exposure during the gestational period has a clear and profound effect on the placental proteome. Air pollution exposure is associated with several types of pathways, both molecular and structural in nature. Future projects should direct their focus on whether these changes on the placental protein level have physiological implications for children's health later in life.

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TABLES

Table 1. Characteristics of selected participants at study (n = 20).

Characteristic	Total population	Low BC exposure	High BC exposure	p-value
Mother				
Age at birth child (years)	29.4 (4.2)	29.2 (3.6)	29.4 (4.9)	0.41
Parity (number of children)	1.8 (1.1)	1.5 (0.5)	2.1 (1.4)	0.25
Alcohol consumption				0.10
Never, n	9 (45.0 %)	3 (30.0 %)	6 (60.0 %)	
Before pregnancy, n	9 (45.0 %)	7 (70.0 %)	2 (20.0 %)	
During pregnancy (occasionally), n	2 (10.0 %)	0 (0.0 %)	2 (20.0 %)	
Gestational diabetes, n	2 (10.0 %)	2 (20.0 %)	0 (0.0 %)	0.47
Hypertension, n	2 (10.0 %)	1 (10.0 %)	1 (10.0 %)	>0.99
Preeclampsia, n	0 (0.0 %)	0 (0.0%)	0 (0.0 %)	>0.99
Education level				>0.99
Low, n (no high school diploma)	2 (10.0 %)	1 (10.0 %)	1 (10.0 %)	
Middle, n (high school diploma)	7 (35.0 %)	4 (40.0 %)	3 (30.0 %)	
High, n (college degree or higher)	11 (55.0 %)	5 (50.0 %)	6 (60.0 %)	
Ambient black carbon exposure (μg/m³)	1.38 (0.60)	0.82 (0.09)	1.94 (0.24)	< 0.001
Placental black carbon load (particles/mm³)	15243.1 (9706.0)	9543.7 (6304.6)	20943.4 (9147.5)	0.007
Child				
Sex				>0.99
Girls, n	10 (50.0 %)	5 (50.0 %)	5 (50.0 %)	
Birth Weight (g)	3395.0 (438.7)	3449.5 (497.8)	3340.5 (389.6)	0.59
Birth Length (cm)	49.9 (2.0)	49.9 (2.6)	50.0 (1.4)	0.94
Gestational age (weeks)	39.8 (1.0)	40.1 (0.9)	39.5 (1.1)	0.19

Characteristics presented as means with standard deviation (SD) or frequency with percentage (%). The two-sided Student's t-test was performed to determine potential differences in participant characteristics (continuous variables) between both exposure groups. The Fisher's Exact test was performed to determine potential differences in the categorical variables between the exposure groups. The p-value indicates whether the characteristic differs between the low and high BC groups.

Table 2. Proteins with a 2-fold higher expression in placental tissue in association with prenatal BC air pollution exposure.

Full protein name	Short name	UniProt accession(s)	Significance	Unique peptides
plakophilin-2	PKP2	Q99959	73.59	2
metalloproteinase inhibitor 3	TIMP3	P35625	32.53	7
keratin, type I cytoskeletal 17	KRT17	Q04695	48.3	6
complement factor H-related protein 1	CFHR1	Q03591	36.8	12
plasma membrane calcium-transporting ATPase 4	ATP2B4	P23634, A0A024R968	53.4	3
collagen alpha-2(IV) chain	COL4A2	P08572, A0A024RDW8	30.41	2
unconventional myosin-Ib	MYO1B	E9PDF6, O43795, B0I1S9	25.73	3
fibrinogen-like protein 1	FGL1	Q08830	41.96	12
platelet glycoprotein Ib beta chain	GP1BB	P13224, A0A140GX60, A0A140GX63	48.79	3
palmitoleoyl-protein carboxylesterase NOTUM	NOTUM	Q6P988	27.65	2
amiloride-sensitive amine oxidase [copper-containing] 1	AOC1	P19801, D3DX01	43.4	18
glia-derived nexin; Serine protease inhibitor	SERPINE2	P07093, A0A024R451	31.12	10
tachykinin-3	TAC3	Q9UHF0, A0A024RB47	29.32	4

Proteins with a ≥ 2-fold expression difference between the low and high black carbon exposure group are listed with their full name, short name and accession(s) as found in the UniProt database. Additionally, the number of unique peptides identified for each protein is provided, together with the significance score computed by the PEAKS software, as ($-10 \times \log_{10}$(p-value)).

Table 3. Results of the STRING functional pathway enrichment analysis on placental proteins with a ≥ 2-fold difference in association with prenatal BC air pollution exposure.

Resource	Pathway ID	Pathway description	Proteins involved	FDR p-value
KEGG	hsa04512	ECM-receptor interaction	COL4A2, GP1BB	0.038
GO Process	GO:0002028	Regulation of sodium ion transport	ATP2B4, PKP2, SERPINE2	0.017
GO Process	GO:0045109	Intermediate filament organization	KRT17, PKP2	0.042
Reactome Pathway	HSA-140877	Formation of Fibrin Clot (Clotting Cascade)	GP1BB, SERPINE2	0.011
Reactome Pathway	HSA-109582	Hemostasis	ATP2B4,GP1BB,SERPINE2,TIMP3	0.012

Abbreviations: ATP2B4, plasma membrane calcium-transporting ATPase 4; COL4A2, collagen alpha-2(IV) chain; ECM, Extracellular Matrix; GO, Gene Ontology; GP1BB, platelet glycoprotein Ib beta chain; KEGG, Kyoto Encyclopedia of Genes and Genomes; KRT17, keratin, type I cytoskeletal 17; PKP2, plakophilin-2; SERPINE2, glia-derived nexin; TIMP3, metalloproteinase inhibitor 3.

FIGURE CAPTIONS

Figure 1. Protein profile heatmap of the 13 proteins with a ≥ 2-fold change in association with black carbon exposure during pregnancy. Cell color represents the log2(ratio) to the average abundance across different samples. Samples are clustered vertically according to sample similarity, which shows a strong clustering pattern for the technical replicates of the same biological sample (R1 and R2). The y-axis shows the 13 proteins with a ≥ 2-fold expression change between the high and low exposed groups. Protein expression levels are shown as colored boxes; red indicates a high expression level and green indicates a low expression level.

Figure 2. Differentially expressed proteins in the placental tissue of women exposed to high levels of black carbon air pollution during pregnancy. Proteins with an at least 2-fold higher expression are depicted in dark orange, proteins with a fold change difference of 1.5 to 2 are either depicted in light orange in case of overexpression, and in green in case of under-expression. Two overexpressed proteins (identified as "dihydropyrimidinase-like 2" and "cDNA FLJ54854 highly similar to Junctional adhesion molecule A") are not represented in this figure, since no official abbreviation has been determined for these targets. Proteins connected with a line represent the significant protein-protein interactions as analyzed with STRING enrichment analysis. Direct inhibitory reactions are shown by a red parallel line, direct activation is depicted with a green arrow.