THE LANCET
Planetary Health

Supplementary appendix

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Supplement to: Bongaerts E, Lecante LL, Bové H, et al. Maternal exposure to ambient black carbon particles and their presence in maternal and fetal circulation and organs: an analysis of two independent population-based observational studies. *Lancet Planet Health* 2022; **6:** e803–11.

Supplemental Appendix

Table of contents

Supplementary Methods

Experimental protocol for BC detection in blood, placental and fetal tissues

Images of the blood samples were collected at room temperature using a Zeiss LSM880 (Carl Zeiss, Oberkochen, Germany) equipped with a femtosecond pulsed laser (810 nm, 120 fs, 80 MHz, MaiTai DeepSee, Spectra-Physics, Santa Clara, CA, USA) tuned to a central wavelength of 810 nm using a Plan-Apochromat 20x/0·8 (Carl Zeiss). Likewise, images from the placental and fetal tissue sections were collected using an EC Plan-Neofluar 10x/0·30 objective (Carl Zeiss) on the above set-up. Two-photon induced white light emission by carbonaceous particles was acquired in the non-descanned mode after spectral separation and emission filtering using 405/10 nm and 550/200 nm band-pass filters. Each blood sample was vortexed and aliquoted at 100 µL per imaging chamber, and ten by ten tile scans were collected 5 µm inwards from the bottom of the imaging chamber (i.e., 170 µm thick 24x24 mm coverslip). The resulting tile scans had a field of view of 4250·96x4250·96 μm^2 containing 100 images with a 5120x5120 pixel resolution and were recorded with a 1·54 µs pixel dwell time at three different locations in the imaging chamber. The placental and fetal tissue sections were imaged using tile scans. The sizes of the recorded tile scans were based on the tissue area covering the section and were recorded with a 1536x1536 pixel resolution and a 4·10 µs pixel dwell time. A minimum of five randomly chosen tissue sections was imaged per sample. The images were acquired by ZEN Black 2·0 software (Carl Zeiss).

To count the number of BC particles in the tile scans recorded for each blood-filled imaging chamber and tissue section, an automated and customised MATLAB program (MATLAB 2010, MathWorks, Natick, MA, USA) was used.[1] First, a peak-find algorithm detects connected pixels above a certain threshold value. For placental and fetal tissue, threshold values of 99·5% and 55% from the highest pixel intensity of the narrow second harmonic generation channel (405/10) and two-photon excited autofluorescence channel (550/200) were used, respectively. For blood, the corresponding threshold values were 80% and 20% from the highest pixel intensity, respectively. These thresholds resulted in highly reproducible values, which were checked manually using Fiji (ImageJ v2·0, open source software, http://fiji.sc/Fiji). Next, the detected pixels in both channels are compared and only the matching ones are used to generate the output image and metrics. The average amount of detected BC particles in blood was normalised for the imaging volume using the focal volume estimated from the spatial resolution of the optical system (810 nm, identical settings, 20x/0·8): $w_x=w_y=0{\cdot}48$ µm and $w_z=2{\cdot}37$ µm, defined as the sizes of the point spread function in the XY-plane (radius of the $1/e^2$ intensity level). In addition, the effectively imaged placental and fetal tissue area was determined from the TPAF image using Fiji. Finally, the total relative number, i.e., the number of detected BC particles per mL and mm^3, was defined for blood and tissue, respectively.

Validation experiments of white light from BC in blood, placental and fetal tissues

Validation experiments were performed using a Zeiss LSM880 with a Plan-Aprochromat 20x/0·8 (Carl Zeiss) suitable for non-linear optical imaging. Optical sectioning in the z-direction throughout the fetal and placental tissue was performed to show tissue embedment of BC particles. Approximately 200 images of each 512 by 512 pixels and with voxels of 1·137x1·137x0·500 μm^3 were acquired throughout the placental and fetal tissue sections using a pixel dwell time of 4·10 µs. In total, a volume of 582·22x582·22x100 μm^3 was imaged. Orthogonal XZ- and YZ-projections were made using Fiji.

The emission fingerprints of the BC particles present in blood and the fetal and placental tissue sections along with two-photon excited autofluorescence of blood and tissue cells were collected under femtosecond pulsed illumination. Note, for this specific experiment, the gain and laser power were decreased to avoid saturation of the emission signal in order to be able to observe the trend of the white light signal over all wavelengths. Accordingly, the emitted signals ranging between 410–650 nm were collected in 9·7 nm bins using the QUASAR thirty-two channel GaASP spectral detector of the LSM880 system. The resulting 1024x1024 lambda image stack with a field of view of 106·50x106·50 μm^2 was detected with a pixel dwell time of 2·05 µs. As a reference, the emission fingerprint of commercially available carbon black nanoparticles (conductive carbon black or CCB; 40 µg/mL; US Research Nanomaterials, Houston, TX, USA) was recorded employing the same conditions.

Following femtosecond illumination, the temporal responses of the emitted signals originating from the BC particles in the blood and tissue samples were detected using the BiG.2 GaASP detector of the LSM880 system. The detector was connected to a time tagging MultiHarp 150 module (PicoQuant, Berlin, Germany) that was synchronised to the pulse train of the MaiTai DeepSee laser. Recordings of 512x512 pixels with a field of view of 156·15x156·16 μm^2 were acquired using a pixel dwell time of 8·19 µs. The instrument response function (IRF) was determined by detecting the response of the laser pulse using a zero-lifetime sample (i.e., potassium dihydrogen phosphate crystals) under identical conditions. As a reference, the temporal response of CCB was recorded using identical settings. All time-correlated single-photon counting measurements were captured and analysed using the SymPhoTime 64 software (PicoQuant). Amplitude-weighted average lifetime values (τ_A) were obtained from double-exponential decay fits.

Supplementary Table 1. Characteristics of the 60 mother-neonate ENVIR*ON*AGE pairs.

Characteristic	Mean (10^{th}-90^{th} percentile) or frequency (%)
Neonate	
Sex	
Girls, n	26 (43·3%)
Boys, n	34 (56·7%)
Ethnicity	
European, n	52 (86·7%)
Non-European, n	8 (13·3%)
Gestational age, weeks	39·6 (38·0-41·0)
Parity	
1	29 (48·4%)
2	20 (33·3%)
≥ 3	11 (18·3%)
Mother	
Age at delivery, years	30·3 (25·0-35·9)
Pre-pregnancy BMI, kg/m^2	24·1 (19·1-31·6)
Education	
Low (no diploma or primary school), n	7 (11·7%)
Middle (high school), n	20 (33·3%)
High (college or university degree), n	33 (55·0%)
Medical problems during pregnancy	
No, n	50 (83·3%)
Gestational diabetes, n	4 (6·7%)
Hypothyroidism, n	2 (3·3%)
Asthma, n	2 (3·3%)
Hypertension, n	1 (1·7%)
Vaginal bleeding, n	1 (1·7%)
Employment during pregnancy	
Manager, n	7 (11·6%)
Professional, n	15 (25·0%)
Technician or associate professional, n	16 (26·7%)
Clerical support worker, n	4 (6·7%)
Service or sales worker, n	12 (20·0%)
Plant or machine operator, n	2 (3·3%)
None, n	4 (6·7%)

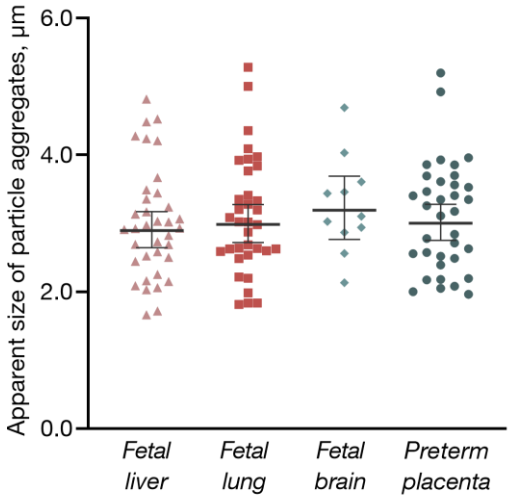
Supplementary Table 2. Characteristics of the 36 mother-fetus SAFeR pairs.

Characteristic	Mean (10^{th}-90^{th} percentile) or frequency (%)
Fetus	
Sex	
Girls, n	18 (50%)
Boys, n	18 (50%)
Fetal age, weeks	13·6 (10·5-18·1)
Mother	
Age at termination, years	25·6 (19·0-37·0)
Pregnancy BMI before termination, kg/m^2	27·2 (18·8-35·7)

Supplementary Note

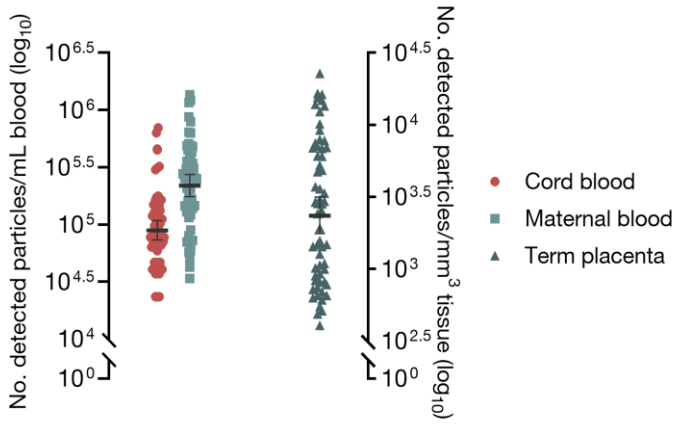
The size distribution of identified BC particles/aggregates in fetal tissues

Concerning the BC particle size, most likely, the particle aggregates consist of various smaller particles which translocated from the mothers' circulation across the placenta to the fetal circulation and organs but this needs to be confirmed in biokinetic follow-up studies It should be noted that the method employed is optically based and therefore is limited by diffraction effects. The measured optical points spread function (PSF) of the used microscopy optical system (810 nm, 0·3 numerical aperture (NA)) is 1·44 µm (radius of the Airy-disk). The diffraction of light puts limits to the particle size determination. While deconvolution can improve size estimations, with the used set-up particles with sizes smaller than the Airy disk diameter (2·88 µm) cannot be determined accurately. Nonetheless, as a very crude approximation, we analyzed the apparent sizes of the particle aggregates of the screened fetal tissue organs. The results are summarized in the figure below. No significant difference in the average apparent particle aggregate size between the different tissues could be determined using one-way ANOVA with Tukey's multiple comparison test. From the analysis it is clear that the mean apparent particle size is not different between the different tissues.

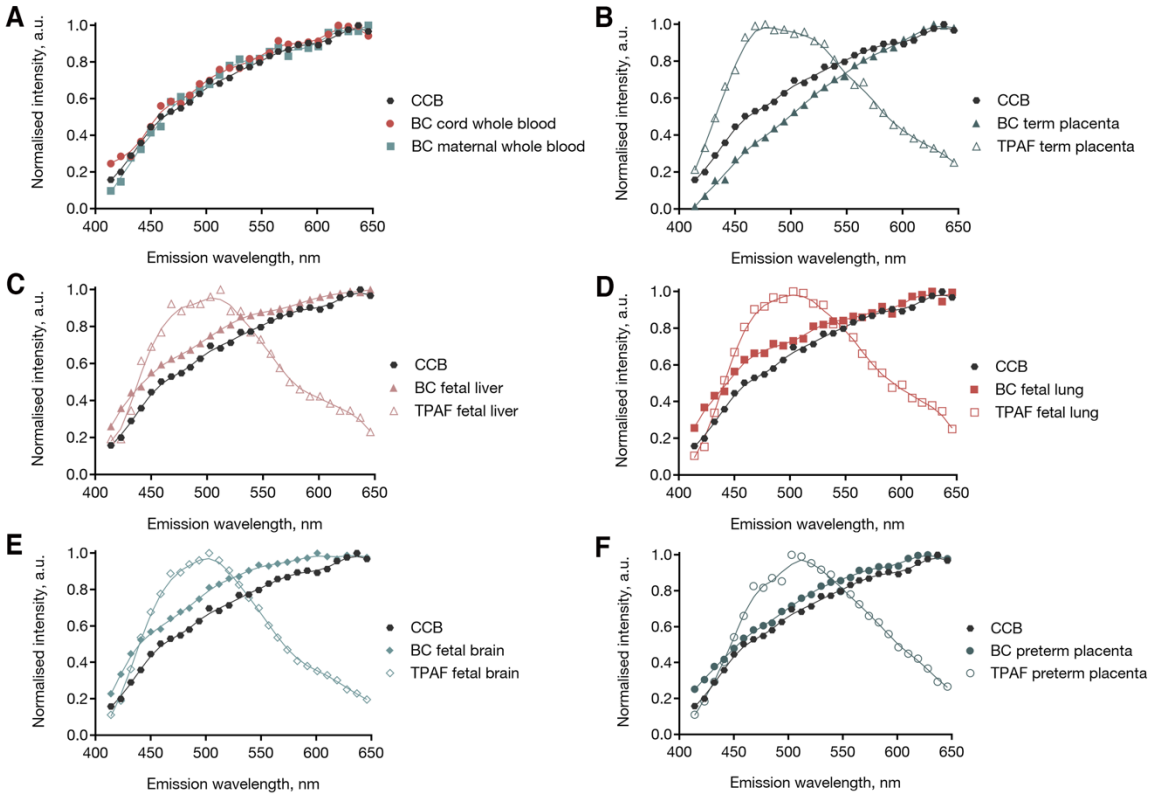


Supplementary Figure S1. Size distribution. The apparent average (SD) BC particle aggregate size measured in fetal liver (n=36), fetal lung (n=36), fetal brain (n=14) and preterm placenta (n=36). The horizontal line marks the mean and the whiskers indicate the corresponding standard deviation. Abbreviations – BC: black carbon; SD: standard deviation.

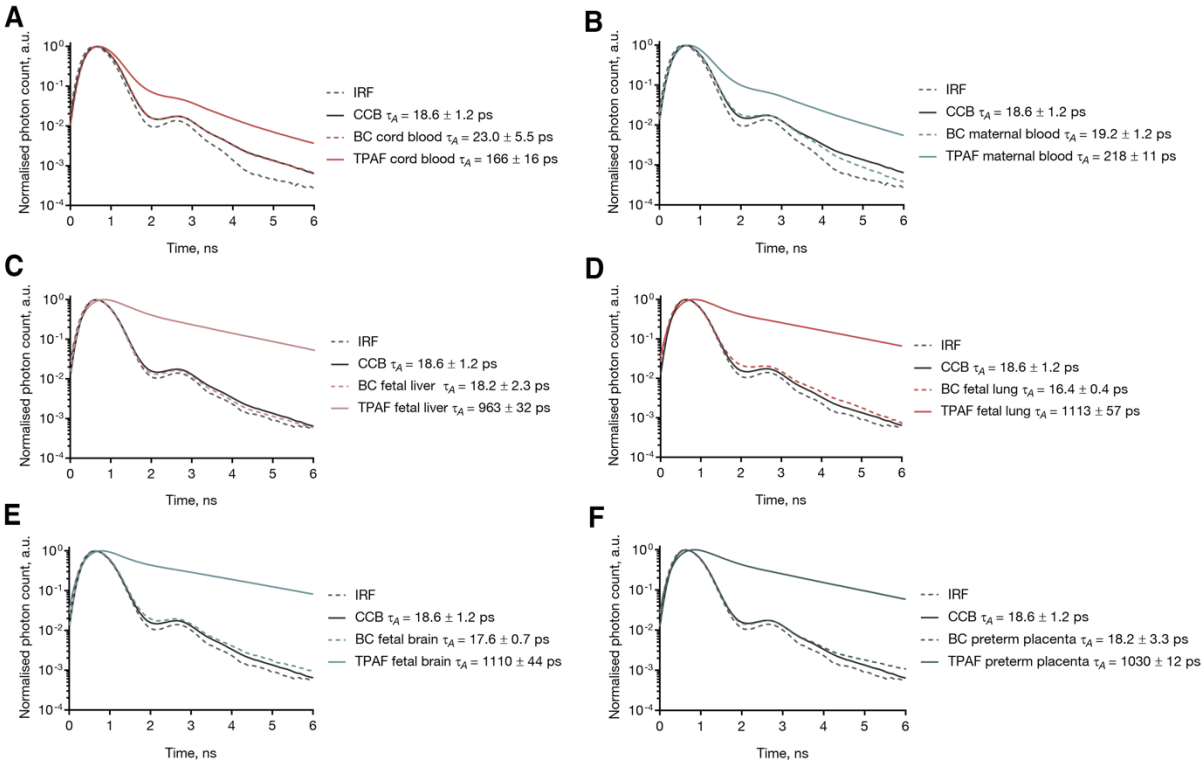
Since the optical resolution depends on the numerical aperture of the employed, one can argue that an objective with higher NA can be employed to gain better insights into the size distribution of the particles. However, screening tissues with a high NA objective would be too time-consuming as data sampling frequency would have to increase accordingly. Moreover, even with a high NA objective the optical resolution is still limited by the diffraction limit of light.



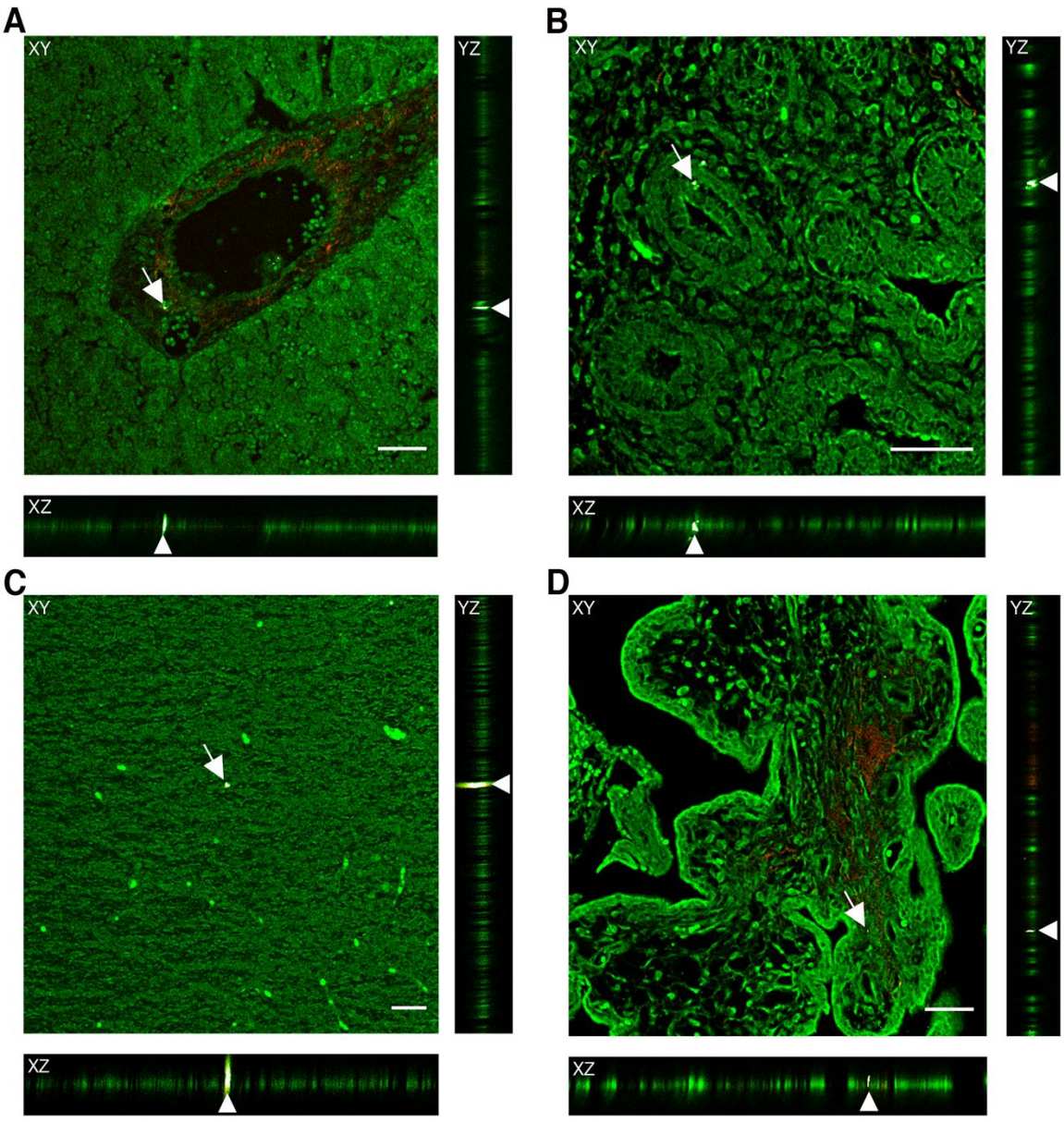
Supplementary Figure S2. Maternal-perinatal BC load. BC particles could be detected in all blood and term placenta samples collected from 60 mother-neonate pairs enrolled in the ENVIR*ON*AGE birth cohort study. The red dots, light blue squares and dark blue triangles indicate the $\log_{10}$-transformed BC load in cord blood, maternal blood and term placental tissue, respectively. The horizontal line marks the geometric mean, and the whiskers indicate the corresponding 95% confidence interval. Abbreviations – BC: black carbon; ENVIR*ON*AGE: ENVIRonmental inluences *ON* early AGEing.



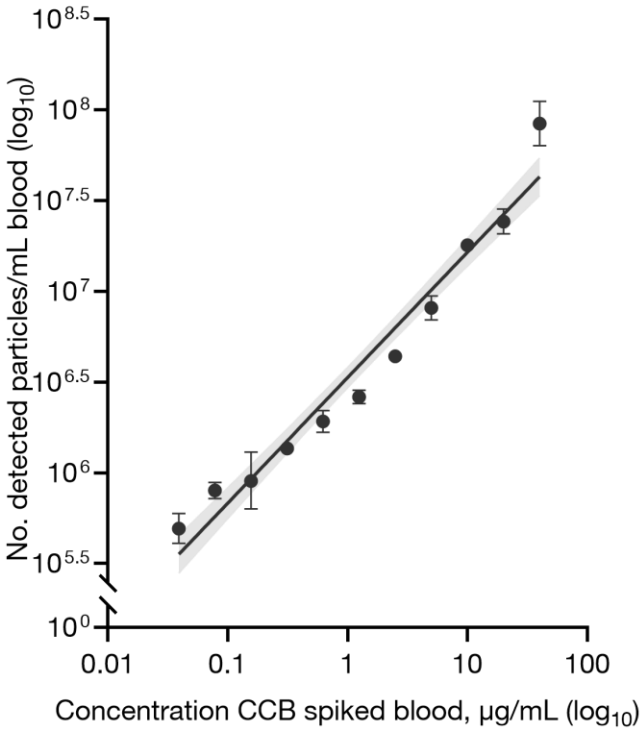
Supplementary Figure S3. Emission profiles. Emission fingerprints measured for the maternal-perinatal samples collected within the ENVIR*ON*AGE study (i.e., maternal and cord blood (**A**) and term placenta (**B**)) and the SAFeR study (i.e., fetal liver (**C**), lung (**D**), brain (**E**), and preterm placenta (**F**)). Carbonaceous particles, including the environmental pollutant BC and commercially engineered CB, generate white light under femtosecond pulsed near-infrared illumination; hence the emission signal stretches the whole visible spectrum. The recorded emission fingerprints of the identified BC particles in blood and placental and fetal tissue show a signal that increases monotonically with wavelength in this range of emission wavelengths as expected (see ref[14]). CCB particles were measured as a reference and confirm the white light emission profile. In contrast, the emission fingerprint of the background signals (i.e., TPAF) of the placental and fetal tissues (**B-F**) consists of a distinct peak around 500 nm after which the signal decreases with wavelength. Abbreviations – BC: black carbon; CB: carbon black; CCB: conductive carbon black; ENVIR*ON*AGE: ENVIRonmental inluences *ON* early AGEing; SAFeR: Scottish Advanced Fetal Research; TPAF: two-photon excited autofluorescence.



Supplementary Figure S4. Lifetime measurements. The peak normalised and reconvoluted traces showing the temporal response of identified BC, reference CCB particles and TPAF measured by time-correlated single photon counting in maternal (**A**) and cord (**B**) blood and fetal liver (**C**), lung (**D**), brain (**E**), and preterm placenta (**F**). The fitted amplitude-weighted average lifetimes (τ_A) of the identified BC and reference CCB particles is non-resolved from the IRF. These results are consistent and validate the carbonaceous nature of the BC particles, as their temporal response is known to be instantaneous. Abbreviations – BC: black carbon; CCB: conductive carbon black; IRF: instrument response function; TPAF: two-photon excited autofluorescence.



Supplementary Figure S5. Fetal tissue embedment of BC particles. XY-images acquired throughout sections of fetal liver (**A**), lung (**B**), brain (**C**), and preterm placenta (**D**) in the Z-direction and corresponding orthogonal XZ- and YZ-projections showing the embedment of a BC particle (white and indicated with white arrowheads) inside the tissue hereby excluding external contamination. Scale bars: 50 µm. Abbreviation – BC: black carbon.



Supplementary Figure S6. Calibration curve. Relation between the added concentration of reference carbon black (CCB) particles in adult whole blood and the amount of particles detected per mL blood. The data are given as geometric mean ± standard deviation (n=3 technical repeats) and fitted linearly (R^2=0.95). Abbreviation – CCB: conductive carbon black.

Supplementary Reference

1 Bové H, Bongaerts E, Slenders E, *et al.* Ambient black carbon particles reach the fetal side of human placenta. *Nat Commun* 2019; **10**: 3866.