The presence of air pollution particulate matter in cryopreserved placental tissue cells

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To the Editor:

There is strong evidence that prenatal air pollution particulate matter (PM) exposure is associated with adverse fetal outcomes, such as restricted fetal growth [1] and an increased risk of preterm birth [2]. Although it remains unclear how inhaled particles into the maternal lung can affect fetal outcomes, it is widely accepted that remote inflammatory processes can result from pro-inflammatory mediators [3], released by airway macrophages during PM phagocytosis. However, translocation of inhaled particles *via* the circulation [4], and impaired placental vascularisation [5] have been implicated.

Using fresh human placentas in London (UK), we recently reported the presence of metal-bearing, air pollution-derived PM in placental phagocytes (macrophage-enriched placental cells, MEPCs), demonstrating the ability of ultrafine PM to translocate to extrapulmonary organs and interact with tissue resident phagocytes [6].

Here, we sought to determine whether this methodology can be applied to stored cryopreserved placenta samples in Hong Kong, a densely populated city with an extremely compact urban plan, where the population is regularly exposed to high levels of traffic-derived air pollution [7].

Healthy never-smoking pregnant women were recruited from the Prince of Wales Hospital, Shatin, Hong Kong from October to December 2019, as part of the "Intestinal Microbiota on Allergy, Growth and Development of the Next Generation in Hong Kong" (SMART Gen HK) birth cohort study. Women provided informed written consent (research ethics committee (REC) reference: CRE-2018.252). Exclusion criteria included oligohydramnios, pre-eclampsia, intra-uterine growth restriction and abnormal placental perfusion.

Annual mean PM_{10} and $PM_{2.5}$ (particles with a 50% cut-off aerodynamic diameter of 10 µm and 2.5 µm, respectively) levels in the districts where participants resided were obtained from the Hong Kong Environmental Protection Department website (https://www.epd.gov.hk/epd/english/top.html).

Placentas were acquired within 2.5 h of delivery. Placental tissue blocks, systematically biopsied from the centre towards the placenta edge, were stored in freezing media (Roswell Park Memorial Institute RPMI-1640, Thermo Fisher Scientific, USA) supplemented with penicillin-streptomycin (Sigma-Aldrich, USA), 10% fetal bovine serum (FBS, STEMCELL Technologies, Canada) and 10% dimethyl sulfoxide (VWR, USA). Samples were stored at −80°C for 3.7±0.9 weeks before processing.

Cryopreserved tissues were thawed at 37°C and processed as previously described [6]. Extracted cells were resuspended in DPBS supplemented with 2% FBS for macrophage enrichment.

MEPCs were extracted as previously described [6, 8], washed in DPBS and resuspended in RPMI medium for analysis by light microscopy.

MEPCs were cytocentrifuged onto microscope slides before staining with Wright Stain Solution (Polysciences, USA) and imaging by light microscopy (×100). 500–1000 randomly selected MEPCs per

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Carbonaceous particles seen in frozen human macrophage-enriched placental cells can be used as a biomarker of personal exposure to combustion-derived particulate matter. The feasibility of using frozen tissues will allow for global comparative studies. https://bit.ly/3yANbRi

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sample were examined, the mean area (μm^2) of black inclusions within each MEPC was quantified using the ImageJ software 1.50i (National Institute of Health, USA) as previously described [9]. Results were compared with the MEPC carbon data from the London cohort [6] (REC-reference: 17/NW/0092).

Data are presented as mean±standard error of the mean (SEM) and compared by t-test. Analyses were performed using Prism 8.00 (GraphPad Software, CA, USA). Results are considered significant at $p<0.05$.

Six healthy never-smoking pregnant women donated their placentas. Subjects resided within 20 km of the Prince of Wales Hospital, with annual mean PM_{10} and $PM_{2.5}$ exposure of 29±0.63 $\mu g \cdot m^{-3}$ and 18±0.63 $\mu g \cdot m^{-3}$, respectively; all women delivered a healthy singleton infant at term by uncomplicated spontaneous or assisted vaginal delivery.

MEPCs had heterogenous morphology typical of macrophages (figure 1a–d). Black inclusions were observed in MEPCs from all samples and were compatible with the appearance of PM identified in MEPCs from healthy women in London [6]. A minimum threshold of ⩾500 cells per participant was selected for quantitative analysis. Five of six samples contained ⩾500 cells. For each sample, 500–1000 cells were randomly selected. Black inclusions were observed in an average of 1% of MEPCs from all women (mean MEPC carbon 0.0044±0.0005 μm^2, figure 1e and f). The black inclusions were irregularly shaped, compatible with appearance of phagocytosed PM in MEPCs from women in London [6], and airway macrophages from healthy children in London [10, 11], REC-reference: 17/EM/0023, (figure 1g and h). Mean MEPC carbon from cryopreserved samples was similar to that previously reported in MEPCs extracted from fresh placentas from women in London (0.0044±0.0009 μm^2, p=0.97, figure 1i) [6]. Exposure of London women was similar to that of women from Hong Kong (modelled annual mean PM_{10} of 27.22±0.60 $\mu g \cdot m^{-3}$ in London *versus* 29.00±0.63 $\mu g \cdot m^{-3}$ in Hong Kong, p=0.09; and modelled annual mean $PM_{2.5}$ of 16.94±0.38 $\mu g \cdot m^{-3}$ in London *versus* 18.00±0.63 $\mu g \cdot m^{-3}$ in Hong Kong, p=0.15) [6].

We previously showed that MEPCs in extrapulmonary tissues are exposed to, and can interact with, translocated traffic-derived nanoparticles. It remains unclear if the concentration of nanoparticles is sufficient to have adverse effects in these distant sites. However, it is widely accepted that the amount of black carbon in airway macrophages reflects longer term PM exposure [12]. In the present study, we sought to demonstrate the feasibility of using cryopreserved placental samples to extract MEPCs.

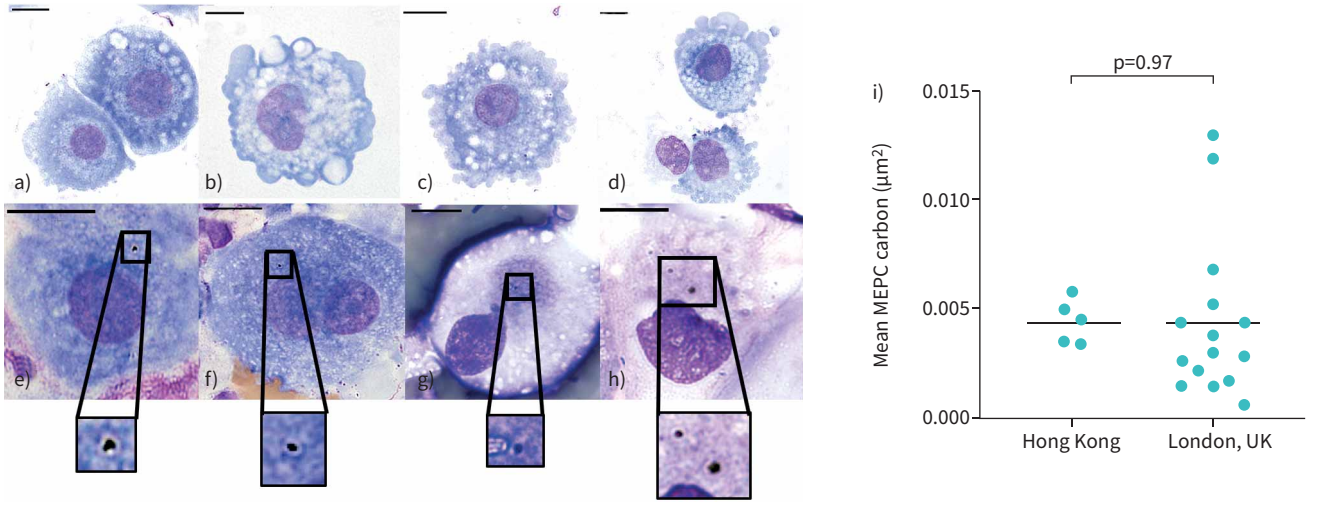

FIGURE 1 a–d) Examples of macrophage-enriched placental cell (MEPC) isolates extracted from cryopreserved placental tissues under light microscopy, their heterogenous morphology (irregular outline, with cytoplasmic vacuolation and lamellipodia) is typical of macrophages. e) and f) Light microscopy images of MEPC isolates from different Hong Kong participants: black inclusions are compatible with those in fresh placental cell isolates previously reported in London, UK (g) [6]. h) Example of phagocytosed particulate matter in an airway macrophage obtained by sputum induction from a healthy child in London [11]. i) Comparison of MEPC carbon from Hong Kong extracted from cryopreserved tissues and London extracted from freshly collected placentas [6]. Mean placental macrophage black carbon was determined from 500 to 1000 cells. Comparison by unpaired t-test. Bars represent mean. Scales bars=10 μm.

Bové *et al.* [13] reported the presence of black carbon in biobanked placental tissues and demonstrated the feasibility of using stored placental tissues to detect black particles. Here for the first time, also using stored tissues, we identified inclusions of carbonaceous particulate matter in MEPCs from women in Hong Kong. We previously showed that these cells have phagocytic capacity [6]; their interactions with particles may have potential to cause adverse health effects, as seen in the lungs where PM phagocytosis by airway macrophages results in release of inflammatory mediators.

There are limitations to this study. First, we did not test for cell viability post-freezing. However, nonviable cells often have altered morphology and do not survive the enrichment process. Second, the small sample size and absence of personal pollutant exposure data is insufficient to assess the association between MEPC carbon loading and PM exposure.

The advantage of using cryopreserved over fresh placental tissue is that it removes the need to process samples immediately, allowing MEPC carbon loading comparison in samples collected from different areas at different times. Compared with MEPC carbon loading in fresh London samples, MEPC carbon loading in Hong Kong samples was of a similar order of magnitude (mean±SEM 0.0044±0.0005 μm^2 in Hong Kong *versus* 0.0044±0.0009 μm^2 in London) [6]. Hong Kong and London have comparable levels of PM, as reported in this study. Indeed, in 2016, the annual mean of $PM_{2.5}$ was 20 $\mu g \cdot m^{-3}$ in Shatin, Hong Kong (https://www.epd.gov.hk/epd/english/top.html), and 13.3 $\mu g \cdot m^{-3}$ in London (https://londonair.org.uk/LondonAir/Default.aspx).

This study suggests that cryopreserved MEPC carbon loading is a promising biomarker to assess associations between maternal exposure and birth outcomes. This technique is suited to studies in pregnant women exposed to burning of biomass fuels, since in many areas of the world over 90% of rural homes use biomass as the primary cooking/heating fuel [14]. Furthermore, airway macrophage carbon loading is higher in women exposed to biomass burning compared with those using cleaner cookstoves [15]. Analysis of extracted MEPCs shows the presence of carbonaceous PM with the appearance of fossil-fuel derived PM. We conclude that MEPC carbon loading in cryopreserved placental tissues is a promising biomarker of exposure of pregnant women to combustion-derived PM.

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Data sharing statement: Study data are available to peer reviewers, and anonymised individual data are available on request to recognised academic institutions, health service organisations or commercial research organisations with experience in medical research.

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