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Ambient black carbon particles in human ovarian tissue and follicular fluid

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ABSTRACT

Evidence indicates a link between exposure to ambient air pollution and decreased female fertility. The ability of air pollution particles to reach human ovarian tissue and follicles containing the oocytes in various maturation stages has not been studied before. Particulate translocation might be an essential step in explaining reproductive toxicity and assessing associated risks. Here, we analysed the presence of ambient black carbon particles in (i) follicular fluid samples collected during ovum pick-up from 20 women who underwent assisted reproductive technology treatment and (ii) adult human ovarian tissue from 5 individuals. Follicular fluid and ovarian tissue samples were screened for the presence of black carbon particles from ambient air pollution using white light generation by carbonaceous particles under femtosecond pulsed laser illumination. We detected black carbon particles in all follicular fluid (n = 20) and ovarian tissue (n = 5) samples. Black carbon particles from ambient air pollution can reach the ovaries and follicular fluid, directly exposing the ovarian reserve and maturing oocytes. Considering the known link between air pollution and decreased fertility, the impact of such exposure on oocyte quality, ovarian ageing and fertility needs to be clarified urgently.

1. Introduction

Infertility is a global and increasing issue, with one in six couples worldwide affected at least once during their reproductive lifetime (WHO). Infertility, defined as the inability to achieve pregnancy after 12 months of regular unprotected sexual intercourse, can be explained by a male factor (e.g., impaired spermatogenesis or sperm motility) and/or female factor (e.g., ovulatory disorders, tubal dysfunction or endometriosis) (Carson and Kallen, 2021; Cox, et al., 1990; Inhorn and Patrizio, 2015). However, in 25–30% of the cases, the cause for infertility cannot be established (Evers, 2002). Several lifestyle factors, including smoking, body mass index and postponed parenthood resulting in advanced maternal age, are known to affect fertility, yet the underlying cause of unexplained cases and increasing rates of infertility needs further investigation (Swan and Colino, 2022; Schmidt, 2006; Kamath and Bhattacharya, 2012).

In recent years, environmental factors as possible causal agents in human infertility have gained attention. For example, air pollution has been proposed to impact human reproductive function (Nieuwenhuijsen, 2014; Carré et al., 2017; Conforti, 2018; Slama, 2013; Kim, 2021). Importantly, multiple observational studies have found significant associations between markers of ovarian reserve [anti-Müllerian hormone (AMH)] and levels of air pollution deduced from spatio-temporal interpolation models based on residential addresses (Kim, 2021; La Marca, 2020; Pang, 2023; Abareshi, 2020). This suggests that exposure to polluted air may disrupt the growth of ovarian follicles and/or accelerate reproductive ageing. However, the exact target organs and mechanisms of toxicity remain unclear. Fertility in women is controlled

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by multiple organs that are involved in the interplay of hormones along the hypothalamus-pituitary-ovary axis to enable the release of mature oocytes. Currently, there are no data to demonstrate that human ovaries and oocytes are directly exposed to air pollution particles. Furthermore, epidemiological studies generally estimate exposures based on spatial–temporal interpolation models, which have low accuracy on an individual level. This lack of data on target organs and exposure levels hampers mechanistic studies that would provide causal proof.

Air pollution is a complex mixture of different constituents which varies, among others, with time, location and the weather. Nowadays, air pollution is recognised as the largest environmental threat to human health, which is particularly true for particulate matter (PM) given its role in increasing pulmonary oxidative stress and inflammation but also due to its link with all-cause mortality, lung cancer, cardiopulmonary disease and stroke (Fuller, 2022; Chen and Hoek, 2020; Rhinehart, 2020; Li, 2020; Nemmar et al., 2004; Nemmar et al., 2013). Black carbon is an important constituent of the fine PM fraction of air pollution and is emitted as an unwanted by-product from the incomplete combustion of fossil fuels, biofuels and biomass. It has been shown that combustion-derived particles are more harmful to human health than PM not generated by combustion (Donaldson, 2005). Accordingly, we consider black carbon as a valuable indicator to evaluate the impact of combustion-derived air pollution on reproductive functions. Black carbon should not be confused with carbon black, which is intentionally manufactured with well-controlled properties. For example, carbon black can be found in vehicle tires and rubber automotive products and ambient carbon black exposure can result from the abrasion of tires and brakes (Pant and Harrison, 2013; Jeong, 2020). Ambient black carbon particles are usually coated with several layers of various toxic co-emitted constituents, including sulphates, nitrates, ammonia, heavy metals, benzene, polycyclic aromatic hydrocarbons, volatile organic compounds (VOCs) and semi-VOCs, thereby bringing potential harm to human health (Chen et al., 2018; Long et al., 2013; Bond, 2006).

In the present study, we investigated the occurrence of black carbon in follicular fluid and ovarian tissue to provide the first exposure assessment on an individual level in a relevant target organ. We demonstrate direct exposure of human ovarian tissue and oocytes to PM during peak reproductive years in every patient studied.

2. Methods

2.1. Study population and sample collection

Ovarian follicular fluid samples were collected from consenting women undergoing infertility treatment (N = 20) at Reproductive Medicine, Karolinska University Hospital, Huddinge, Sweden, in 2015. The recruitment was continuous and only patients that did not speak Swedish were excluded. Women underwent standard ovarian stimulation and oocyte pick-up via *trans*-vaginal ultrasound guided needle-aspiration from both ovaries. Leftover ovarian follicular fluid, which would have otherwise been discarded, was collected for the current study. After the exclusion of blood-contaminated aliquots, the clear liquid was pooled per patient, centrifuged to pellet the cellular debris, and frozen in −80 °C until analysis. Data on patient age and body mass index were retrieved from medical records, and then the samples were completely anonymised. Aliquots were used to quantify the presence of black carbon particles. Procedure blanks were prepared by aspirating 5 mL PBS through oocyte aspiration needles (N = 2) in triplicates to study the possible presence of black carbon in the laboratory setting. The study was approved by Swedish Ethical Review Authority (license number 2014/1943–31/2).

Ovarian tissue samples were collected from consenting gender reassignment patients (N = 5) at Karolinska University Hospital Huddinge in 2022. The recruitment was continuous and included all Swedish-speaking patients scheduled for bilateral oophorectomy as a part of their gender-reaffirming surgery, and the tissue that would have been discarded was collected for the current study. The tissue was picked up fresh at the operation theatre and transported to the research laboratory in warm PBS within 10 min. A tissue slice was cut across the middle of the ovary and fixed in 4% formalin for 24 h at 4 °C, transferred to 70% ethanol, embedded in paraffin and processed into 4 μm tissue sections. Some sections were stained with haematoxylin and eosin to control for the presence of follicles of normal morphology. For the current study, five patients were selected based on the presence of histologically confirmed normal ovarian structures and follicles. Unstained sections were used to assess the presence of black carbon particles. Data on age, hormonal treatments and body mass index were collected from medical records, and the samples and data were then pseudonymised and registered to Stockholm Medical Biobank. The study was approved by the Swedish Ethical Review Authority (license number 2015/798–31).

2.2. Experimental protocol for black carbon detection in follicular fluid and ovarian tissue

Black carbon particles from ambient exposure present in follicular fluid and ovaries were detected using a specific and sensitive detection technique based on the non-incandescence-related white light generation of the particles under femtosecond illumination, as previously described (Bové, 2016; Bongaerts, 2022; Bové, 2019). We previously confirmed the carbonaceous nature and tissue embedment of the identified black carbon particles using rigorous validation experiments (Bongaerts, 2022; Bové, 2019).

Images of the follicular fluid were collected at room temperature using an inverted Zeiss LSM880 confocal microscope (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 of the ovarian 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 follicular fluid sample was vortex and aliquoted at 50 μL per imaging chamber, and 10 by 10 tile scans were collected 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 4250.96 × 4250.96 μm^2 containing 100 images with a 5120 × 5120 pixel resolution and were recorded with a 1.54 μs pixel dwell time at three different locations in the imaging chamber (N = 3 technical replicates). The ovarian tissue sections were also imaged using tile scans. The size of the recorded tile scan was based on the tissue area covering the section and was recorded with an 1824 × 1824 pixel resolution and a 2.3 μs pixel dwell time. A minimum of five randomly chosen tissue sections was imaged per sample. Additional detailed images were collected from cortical and medullar regions of the ovaries to investigate a differential black carbon load. All images were acquired by ZEN Black 2.0 software (Carl Zeiss). To count the number of black carbon particles in the tile scans recorded for each follicular fluid-filled imaging chamber and ovarian tissue section, an automated and customised MATLAB program (MATLAB 2010, Mathworks, Natick, MA, USA) was used (Bové, 2019). First, a peak-finding algorithm detects connected pixels above a specific threshold value. For follicular fluid, threshold values of 80% and 20% from the highest pixel intensity of the narrow 405/10 nm and broad 550/200 nm channels were used, respectively. For ovarian tissue, the corresponding thresholds were 99.5% from the highest pixel intensity for both detection channels. These thresholds resulted in highly reproducible values, which were checked manually using Fiji (ImageJ v2·0, open-source software, https://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 black carbon particles in follicular fluid 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): $w_x = w_y = 0.48$ μm and $w_z = 2.37$ μm, defined as the sizes of the point spread function in the XY-plane and along the optical axis (z-axis) (radius at the $1/e^2$ intensity level). In addition, the effectively imaged ovarian tissue area was determined from the TPAF image using Fiji and multiplied with the tissue thickness (i.e., 4 μm) to obtain the tissue volume. Finally, the total relative number, i.e., the number of detected black carbon particles per mL and mm^3, was defined for follicular fluid and ovarian tissue, respectively.

Validation experiments were performed using a Zeiss LSM880 with a Plan-Aprochromat 20x/0.8 (Carl Zeiss). Optical sectioning in the z-direction throughout the ovarian tissue was performed to show tissue embedment of black carbon particles. Approximately 150 images of each 512 × 512 pixels and with voxels of 1.31 × 1.31 × 0.5 μm^3 were acquired throughout the ovarian tissue using a pixel dwell time of 4.10 μs. In total, a volume of 671.54 × 671.54 × 74.5 μm^3 was imaged. Orthogonal XZ- and YZ-projections were acquired using Fiji. The emission fingerprints of the black carbon particles present in follicular fluid and ovarian tissue sections along with TPAF 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 and 650 nm were collected in 9.7 nm bins using the QUASAR thirty-two channel GaASP spectral detector of the LSM880 system. The resulting 512 × 512 lambda image stack with a field of view of 212.55 × 212.55 μm^2 was detected with a pixel dwell time of 1.54 μs. As a reference, the emission fingerprint of commercially available carbon black nanoparticles (conductive carbon black or CCB; 40 μg/mL; US Research Materials, USA) was recorded employing the same conditions.

2.3. Statistical analysis

Patient characteristics (age, BMI) were normally distributed as tested by the Shapiro-Wilks test and are summarised as average and SD. Black carbon load data are represented as average (SD) and were analysed using the GraphPad software (GraphPad Prism 8, GraphPad Software Inc., USA). We expressed differences in black carbon particle load between the cortex and medulla of the ovaries as a fold change and analysed them using the two-sided paired *t* test.

3. Results

To study the translocation of carbonaceous air pollution particles towards the human ovaries, we screened 20 follicular fluid samples and 5 ovarian tissue sections for the presence of black carbon particles using white light generation under femtosecond pulsed laser illumination as described in the Methods section. Information about the sample donors is given in Table 1. The gender reassignment patients were on average 29.2 years old with an average BMI of 25.1, while the infertility treatment patients were 33.5 years old with an average BMI of 23.9. These values are close to the population average BMI of 24.8 for women in Sweden around the same time period (i.e., 2016–2017) (Statistiska Centralbyrån, 2018).

We detected black carbon particles from ambient air pollution exposure in all follicular fluid and ovarian tissue samples screened in a label-free and biocompatible manner. Black carbon particles were present in follicular fluid samples with an average (SD) particle count of 5.8 × 10^5 (5.3 × 10^5) black carbon particles per mL fluid (Fig. 1). The average follicular fluid black carbon load ranged from (min–max) 1.2 × 10^5 to 2.4 × 10^6 particles per mL fluid. In contrast, procedure blanks collected from sampling PBS with an aspiration needle did not contain any black carbon particles.

In addition, black carbon particles could be detected in all screened ovarian tissue sections with a differential black carbon load between the cortical and medullary regions (Fig. 2). On average (SD), we detected 5.0 × 10^3 (4.8 × 10^3) and 1.9 × 10^4 (1.2 × 10^4) particles per mm^3 tissue in the cortex and medulla of the ovaries, respectively. The particle load in the medulla was on average 3.8-fold higher ($p = 0.0021$) compared to the ovarian cortex. Histologically particles were found both among the non-growing follicles of the cortex, that constitute the ovarian reserve, and inside growing antral follicles (Fig. 2).

Embedment of the black carbon particles in the ovarian tissue samples excludes external contamination (Fig. 3**A**) and the recorded emission fingerprint confirms the carbonaceous nature of the detected signal (Fig. 3**B**). Both commercially engineered carbon black particles (used as a reference) and the detected black carbon particles in follicular fluid and ovarian tissue generated a white light signal that stretches the visible spectrum under femtosecond pulsed illumination. By contrast, the emission fingerprint of the background signals of the ovarian tissue consisted of a distinct peak that did not continuously range over all wavelengths. This spectral characteristic (i.e., emission signal stretching the visible spectrum) allows for the discrimination of combustion-derived particles from non-carbonaceous particles (Aslam and Roeffaers, 2021).

Table 1
Patient characteristics.

Patient type	*N*	*Age, years (mean ± SD)*	*BMI, kg/m² (mean ± SD)*	*Hormonal treatment*
Gender reassignment	5	29.2 ± 6.9	25.1 ± 5.5	Androgens
Infertility treatment	20	33.5 ± 4.4	23.9 ± 2.9	Gonadotropins

4. Discussion

Recent observational studies have suggested a link between exposure to ambient air pollution and reduced fertility (Nieuwenhuijsen, 2014; Carré et al., 2017; Conforti, 2018; Slama, 2013). For example, PM exposure has been linked to decreased ovarian reserve (Kim, 2021; Gaskins, 2019), longer time to pregnancy (Li, 2021) and a lower success rate in assisted reproductive technology (Legro, 2010) on a population level. The mechanisms are unclear but could involve polycyclic hydrocarbons, heavy metals, phthalates, bisphenols and other endocrine disrupting chemicals contained in PM (Takeda et al., 2004; Okamura, 2004; Kizu, 2003; Quintana-Belmares, 2018; Kanellopoulos et al., 2021), which could possibly affect ovarian steroidogenesis and folliculogenesis (Bellavia, 2023; Björvang, 2022). The mechanism could also include direct particle toxicity leading to the generation of reactive oxygen species that cause, for example, the formation of DNA adducts and genetic modifications (Møller, 2014; Cho, 2005).

In this study, we determined individual exposures to combustion-derived air pollution by visualising and quantifying black carbon particles from ambient air pollution in adult fertile age human ovarian samples (i.e., follicular fluid and ovarian tissue). Studies demonstrating that air pollution particles could reach and directly expose the ovaries have been missing. By showing, for the first time, the presence of black carbon particles in ovaries, we propose direct particle toxicity in ovaries as a potential mechanism to link air pollution exposure with infertility which should be scrutinized in follow-up studies.

The ovary is a vital organ for female reproduction with two main tasks: steroidogenesis and folliculogenesis. The prenatally formed immature oocytes reside in primordial follicles in the ovarian cortex. During folliculogenesis, growing follicles migrate towards the medulla and a liquid-filled antrum forms inside the granulosa cell layers of the follicle. Antral follicles recruit theca cells from the surrounding stroma, become gonadotropin responsive and start secreting steroids as they grow further. Numerous blood vessels carry blood to the growing antral follicles and contribute to the formation of follicular fluid, which forms

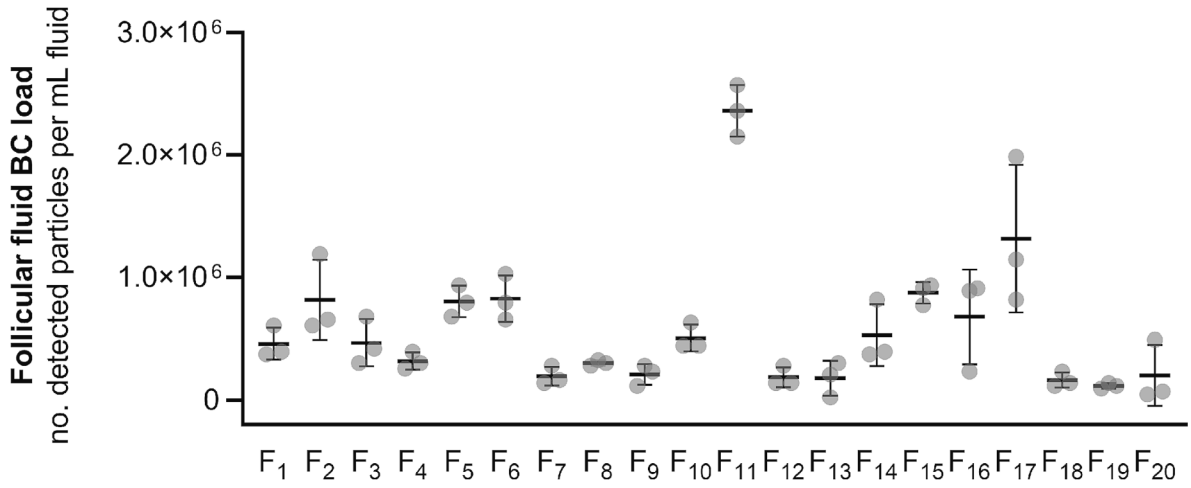

Fig. 1. Follicular fluid black carbon load. Black carbon particles were detected in all screened follicular fluid samples collected from 20 women seeking infertility treatment (patient number $F_{1\text{-}20}$). The data are given as mean (SD) (n = 3 technical repeats).

the immediate microenvironment where the oocytes mature (Rodgers and Irving-Rodgers, 2010). Hence both ovarian tissue and follicular fluid serve as representative matrices to study contaminants that could affect fertility. We used our biocompatible and label-free white light technique for the detection of black carbon particles in these samples. This technique enables direct visualisation of the particle distribution in the ovarian tissue without the need for additional sample preparation. We found particles both in the cortex among the non-growing follicles of the reserve and in growing antral follicles. In addition, we found a higher black carbon load in the ovarian medulla compared to the cortical region, which could possibly be explained by the difference in stromal texture and level of vascularization (Fig. 2). These results suggest that human ovarian follicles are exposed to air pollution particles at all stages of maturation.

Follicles are surrounded by a basement membrane that separates the stroma and theca cell layers from the granulosa cells and the oocyte. This membrane appears to provide very little protection towards contaminants. For example, several observational studies have detected similar concentrations of endocrine disrupting chemicals (e.g., bisphenol A and organochlorine pesticides) in serum and follicular fluid, showing that contaminants easily cross the follicular membrane (Björvang, 2022; Ikezuki et al., 2002). Here, we show that even particles can cross the follicular basement membrane, as black carbon was detected both in the ovarian tissue and follicular fluid. The levels were similar to previously measured particle loads in full-term placentas and maternal whole blood, respectively (Bongaerts, 2022; Bové, 2019). Since black carbon can act as a carrier for co-emitted combustion-derived toxic substances (e.g., heavy metals and VOCs) as well as endocrine disrupting chemicals (e.g., phthalates and bisphenols), their potential harm to the human body is likely a combination of particle toxicity and chemical toxicity (Quintana-Belmares, 2018; Salgueiro-González et al., 2015; Abbas, 2019).

While no studies have focused on black carbon and ovarian follicles, several experimental studies have investigated nanoparticle toxicity in females. For example, human (Simon, 2017; Liu, 2010) and rat (Stelzer and Hutz, 2009) granulosa cells can internalise nanoparticles (e.g., carbon black, TiO_2 and Au nanoparticles), and a study in mouse follicular cells found a correlation between the internalised CeO_2 particles and DNA damage in oocytes (Preaubert, 2016). In addition, *in vivo* studies in rodents have elucidated the distribution of nanoparticles in ovarian cells and tissues after intragastrical and intravenous particle exposure (Zhao, 2013; Schädlich, 2012; Santacruz-Márquez et al., 2021). Although there is a lack of studies evaluating particle accumulation in human ovaries, the above studies suggest that particles could reach the ovarian cells after circulation in the bloodstream. In this regard, we have recently shown the presence of black carbon particles from ambient exposure at similar levels in whole maternal blood (Bongaerts, 2022), which further substantiates our hypothesis that particle accumulation within the ovaries might be regulated by the vasculature. Future studies should investigate the kinetics and distribution of these particles in the human body, including the reproductive system. Such studies could also attempt to correlate particle levels for instance to markers of DNA damage and/or oxidative stress. This will help to increase our understanding of mechanisms controlling particle distribution and toxicity as well as their role in reduced fertility.

While the findings of this work broaden our understanding of the possible adverse outcome pathways underlying the suggested link between air pollution exposure and reduced fertility, they should also be considered in the context of the limitations of this work. First, only a limited sample set (i.e., 20 follicular fluid and 5 ovary tissue samples) was used to assess the presence of ambient black carbon particles in the ovaries. Second, no information on the participants' residential exposure was available due to anonymity, hence we were not able to study the relationship between the ovarian black carbon particle load and the participants' ambient black carbon exposure, vicinity to highly trafficked roads, or other individual activity patterns. Finally, the sample size was too limited to study associations between exposure and reproductive outcomes. However, this study showed the presence of ambient combustion-derived particles in the female reproductive system. These findings provide valuable information to continue investigating how air pollution, in particular combustion-derived particles, may directly impact human fertility through toxicity in ovaries. Follow-up studies should be carried out in larger cohorts that allow an analysis of correlations between black carbon and ovarian function and fertility, as well as an investigation of possible mechanisms. In addition, it will be important to compare populations in different geographical locations characterised by varying levels of PM in the air.

In conclusion, this study proves the presence of ambient black carbon particles in follicular fluid and ovarian tissue. The evidence of combustion-derived particle translocation towards the ovaries might represent a link between reduced fertility rates and increased air pollution levels. Collectively, our study points to the need to detailed hazard characterization in ovaries in order to safeguard women's fertility and the quality of oocytes that represent the next generation.

CRediT authorship contribution statement

Eva Bongaerts: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Visualization. **Katariina Mamia:** Investigation. **Ilmatar Rooda:** Investigation. **Richelle D. Björvang:**

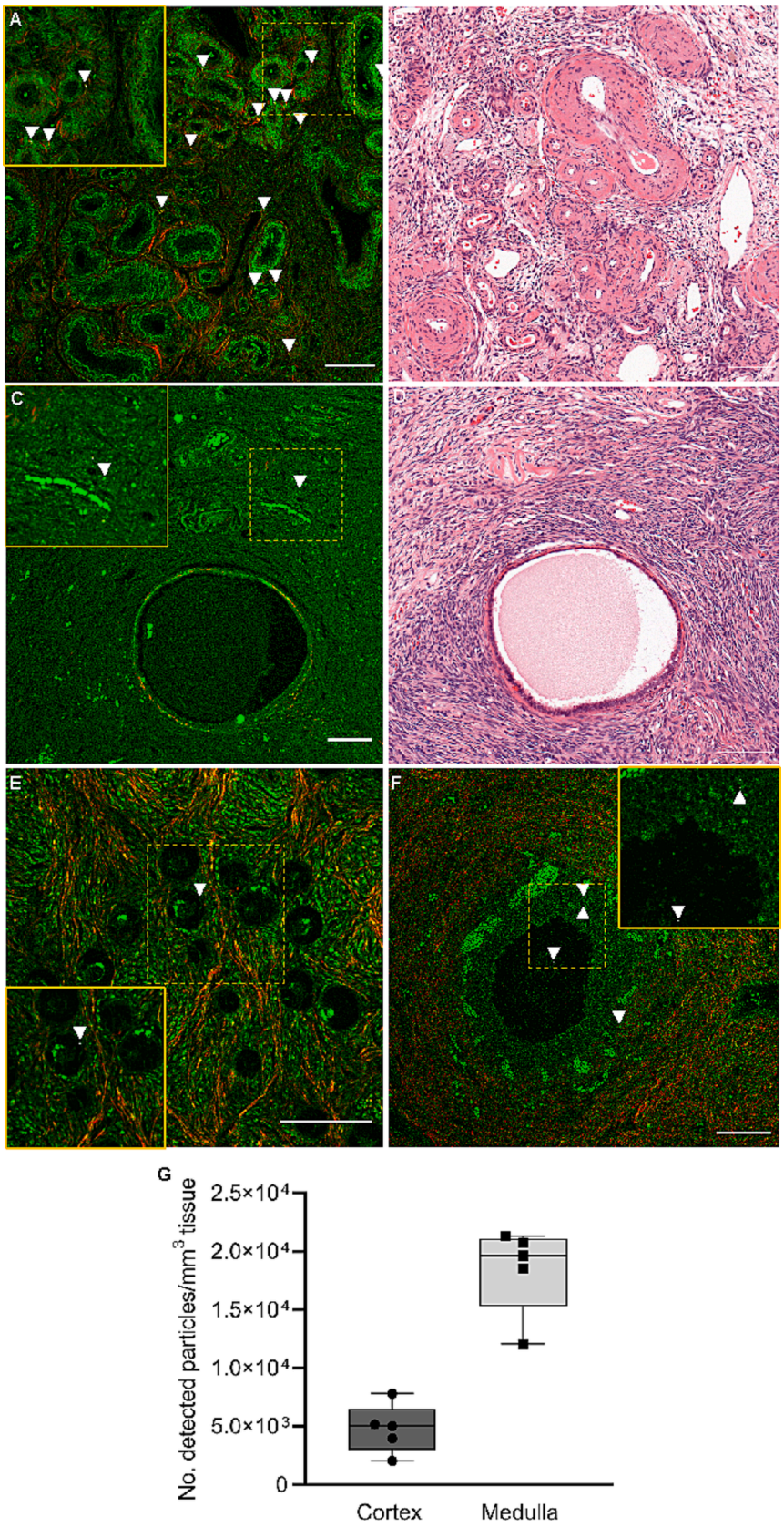

Fig. 2. Ovarian black carbon load. Presence of ambient black carbon particles (white dots further indicated with white arrowheads) in the medullary (A) and cortical (C) region of the human ovaries. Second harmonic generation by collagen (red, emission 400–410 nm) and two-photon excited autofluorescence from ovarian cells (green, emission 450–650 nm) are simultaneously detected. Haematoxylin and eosin staining of representative regions in the medullar (B) and cortical (D) region of the human ovaries showing the typical higher vascularization of the medulla compared to the cortex. (E) Black carbon particle inside a non-growing follicle of the cortical ovarian reserve. (F) Multiple black carbon particles inside a growing antral follicle. (G) Box plot showing the number of detected black carbon particles in medulla and cortex of human ovarian tissue samples collected from 5 patients (patient number O_{1-5}). The line depicts median, box interquartile range, and whiskers the min-max. Scale bars: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

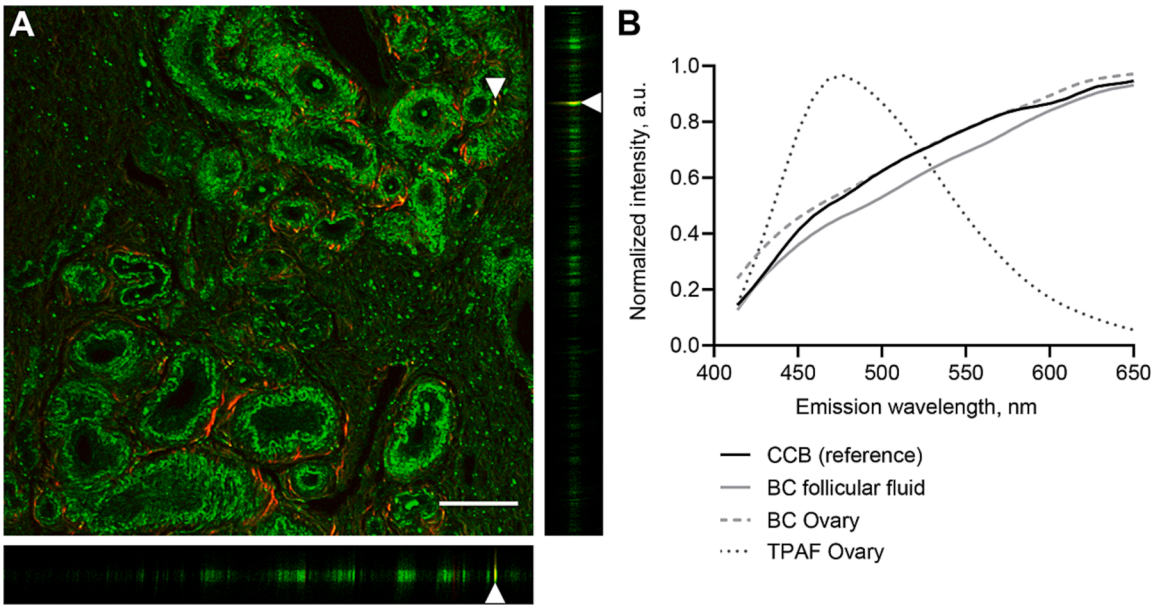

Fig. 3. Tissue embedment and emission fingerprint. (A) XY-images acquired throughout the ovary in the Z-direction and corresponding orthogonal XZ- and YZ-projections showing the intra-ovarian tissue embedment of a black carbon particle (white and indicated with a white arrowhead) hereby excluding external contamination. Second harmonic generation by collagen (red, emission 400–410 nm) and two-photon excited autofluorescence from ovarian cells (green, emission 450–650 nm) are simultaneously detected. (B) Emission fingerprints were recorded for the follicular fluid and ovary samples. Carbonaceous particles, including the environmental pollutant black carbon and commercially engineered carbon black [i.e., conductive carbon black (CCB)], generate white light under femtosecond pulsed near-infrared illumination; hence the emission signal stretches the whole visible spectrum, as shown in the recorded emission fingerprint. In contrast, the emission fingerprint of the background signals (i.e., two-photon excited autofluorescence, TPAF) of the ovarian tissue consists of a distinct peak around 480 nm, after which the signal decreases with wavelength. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Investigation. **Kiriaki Papaikonomou:** Investigation. **Sebastian B. Gidlöf:** Investigation. **Jan I. Olofsson:** Investigation. **Marcel Ameloot:** Methodology. **Ernesto Alfaro-Moreno:** Conceptualization. **Tim S. Nawrot:** Conceptualization, Methodology, Supervision. **Pauliina Damdimopoulou:** Conceptualization, Methodology, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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