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December 24, 2019

Honorable Freda L. Wolfson, Chief Judge
United States District Court
Clarkson S. Fisher Building & US Courthouse
402 East State Street
Trenton, NJ 08608

Re: ***In Re: Johnson & Johnson Talcum Powder Products Marketing, Sales Practices and Products Liability Litigation*** **(MDL No. 2738)**

Dear Chief Judge Wolfson:

The PSC writes to bring to the Court's attention four peer-reviewed publications that have been published since the *Daubert* hearing and that support the PSC's general causation experts' opinions that Johnson & Johnson's talcum powder products can cause ovarian cancer:

- Steffen, JE., et al. *Serous Ovarian Cancer Caused by Exposure to Asbestos in Cosmetic Talc Powders – A Case Series Serous Ovarian Cancer Caused by Asbestos in Cosmetic Talc*. Journal of Occupational and Environmental Medicine. DOI: 10.1097/JOM.0000000000001800. (published ahead of print) (Dec. 23, 2019) (attached as Exhibit A). Investigators, including PSC experts Drs. Longo and Rigler, reported on 10 cases of serous ovarian cancer among users of Johnson & Johnson's asbestos-containing talcum powder products. They performed an asbestos exposure assessment during talc application. The investigators analyzed the surgical tissue of the patients as well as the talc containers used by the patients. Platy talc was found in the tissue of 9 out of 10 cases; fibrous talc was found in 8/10 cases; tremolite and/or anthophyllite asbestos was found in 8/10 cases. The asbestos fibers found in the cosmetic talc containers matched those found in tissues. The estimated inhaled asbestos dose ranged from 0.38 to 5.18 fiber years. None of the cases reported in the series had any known history of alternative asbestos exposure. The study provides evidence that the inhaled asbestos/fibrous talc from the application

of cosmetic talc can cause ovarian cancer. "The unique combination of the types of asbestiform minerals detected in cancerous tissue and 'cosmetic' talc is a fingerprint for exposure to asbestos-containing talc."

- O'Brien, KM, et al. *Genital Powder Use and Risk of Ovarian Cancer: A pooled analysis.* ASPO Abstracts (American Society of Preventive Oncology). Dec. 17, 2019 (attached as Exhibit B): One of the Johnson & Johnson Defendants' primary arguments in support of their position that the epidemiologic literature does not support causation is that the cohort studies do not show a statistically significant increased risk between genital powder use and ovarian cancer, and therefore, are inconsistent with other study designs. The National Cancer Institute's Ovarian Cancer Cohort Consortium conducted a pooled data study from the four large cohort studies. The data included 2,073 cases of ovarian cancer. The investigators observed that there is a statistically significant 9% increase in ovarian cancer with ever powder use, compared to never use (hazard ratio [HR] = 1.09, 95% confidence interval [CI] = 1.00, 1.20). "The strongest association was observed among women with patent reproductive system, e.g. had a uterus and had not had tubal ligation, at the time powder exposure was assessed (HR=1.15, 95% CI: 1.03, 1.29). There were no clear differences by ovarian cancer subtype." The 11 investigators who conducted the study are from institutions such as the NCI, Harvard Medical School, Johns Hopkins University, among others, and their work was funded by the NCI.

- Mandarino, A., et al. *The effect of talc particles on phagocytes in co-culture with ovarian cells.* Environmental Research 180 (2020) 108676 (attached as Exhibit C): The investigators in an *in vitro* study evaluated the immunotoxic effect of talc as compared to a control. Using macrophage cells, similar to those used by Dr. Saed, the investigators "found that murine ovarian surface epithelial cells (MOSEC), a prototype of certain forms of ovarian cancer, were present in larger numbers after co-culture with macrophages treated to a combination of talc and estradiol than to either agent alone or vehicle. Control particles did not have this effect. Co-exposure of macrophages to talc and estradiol led to increased production of reactive oxygen species and changes in expression of macrophage genes pertinent in cancer development and immunosurveillance." These results, from independent scientists from Harvard University, the University of Rochester, and Brown University, are consistent with and support Dr. Saed's research and opinions regarding the biological effects of Johnson's Baby Powder in cell cultures.

- McDonald, SA., et al. *Migration of Talc from the Perineum to Multiple Pelvic Organ Sites: Five Case Studies With Correlative Light and Scanning Electron Microscopy*. Am J Clin Pathol 2019;XX:1–18 (attached as Exhibit D). The investigators reported data from five patients with documented perineal talcum powder use. In each instance involving exposed patients, talc (talc particles and in some instances, talc fibers (i.e., fibrous talc)) was documented by polarized light microscopy and scanning electron microscopy in multiple pelvic sites distant from the perineum. These sites included pelvic region lymph nodes, cervix, uterine corpus, fallopian tubes and ovaries. The existence of morphologically demonstrated talc in multiple pelvic organ sites, including pelvic tissues and lymph nodes simultaneously, reported in this publication has not been reported in the literature previously and confirms the "biologic potential of talc, its inflammatory potential, and its migration via pelvic lymphatics from the perineum." This publication supports the opinions of each of the PSC's general causation experts that talcum powder can migrate from the perineum to the upper genital tract.

Thank you for your consideration of these additional scientific publications. Should the Court have any questions or require additional information, please let us know. We hope you have a happy holiday.

Very truly yours,

/s/ Michelle A. Parfitt
Michelle A. Parfitt

/s/ P. Leigh O'Dell
P. Leigh O'Dell

cc: All counsel of record via ECF notification

Exhibit A

OPEN

Journal of Occupational and Environmental Medicine, Publish Ahead of Print

DOI: 10.1097/JOM.0000000000001800

Serous Ovarian Cancer Caused by Exposure to Asbestos in Cosmetic Talc Powders – A Case Series

Serous Ovarian Cancer Caused by Asbestos in Cosmetic Talc

Joan E. Steffen[1], B.A.; Triet Tran[1], B.A., B.S.; Muna Yimam[1], B.S.; Kate M. Clancy[1,2]; Tess B. Bird[1,3], DPhil; Mark Rigler[4], PhD; William Longo[4], PhD; David S. Egilman[1,5], M.D., M.P.H

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Authors' contributions:

Joan E. Steffen contributed to the conception and design of the work; the acquisition, analysis and interpretation of the data; drafting and editing the work; and final approval of the version to be published. Ms. Steffen contributed to medical record review for the patients, dose calculations, and literature review and analysis. She agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Triet Tran contributed to the conception and design of the work; the acquisition, analysis and interpretation of the data; drafting and critically reviewing the work; and final approval of the version to be published. He collaborated with Joan Steffen, Muna Yimam and Dr. Egilman on dose calculations and also contributed to the literature review and analysis. He also worked with Dr. Egilman on the dose-response risk assessment. He agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Muna Yimam contributed to the analysis and interpretation of the data; drafting and editing the work; and final approval of the version to be published. She also contributed with Triet Tran, Joan Steffen, and Dr. Egilman on dose calculations. She agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Kate M. Clancy contributed to the analysis and interpretation of the data; drafting and editing the work; and final approval of the version to be published. She also contributed to the literature review and analysis. She agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Tess B. Bird contributed to the analysis and interpretation of the data; drafting and editing the work; and final approval of the version to be published. She also contributed to the literature review and analysis. She agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Mark Rigler contributed to the conception and design of the work; the acquisition, analysis and interpretation of the data; critically revising and editing the work; and final approval of the version to be published. Dr. Rigler performed the tissue analyses for asbestos and talc. He also contributed to the literature review and analysis. He agrees to be accountable for all aspects of the work in ensuring that

questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

William Longo contributed to the conception and design of the work; the acquisition, analysis and interpretation of the data; critically revising and editing the work; and final approval of the version to be published. Dr. Longo performed analysis of talc powder for asbestos (reported for Case #3). He agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

David S. Egilman contributed to the conception and design of the work; the analysis and interpretation of the data; drafting and critically revising the work; and final approval of the version to be published. Dr. Egilman examined and interviewed the living patients, contributed to medical record review and performed patient dose calculations. He also contributed to the literature review and analysis. He agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Acknowledgements: We thank Sander Greenland for his review of our risk model and dose calculation equations. Any errors are our responsibility

Funding: Plaintiffs' attorneys in litigation against Johnson & Johnson (Ingham et. al. vs. Johnson & Johnson et. al.) paid for tissue analysis for talc and asbestos in patient tissues. They also paid for travel costs and time spent examining and interviewing patients. There was no outside funding for work on this manuscript.

Institution and Ethics approval and informed consent: There was no requirement for ethics review or institutional review board approval because this research was not experimental and was originally conducted pursuant to a lawsuit. Informed consent was obtained from all living patients. For one deceased patient (Case #8), consent was obtained from the surviving spouse. For the remaining two deceased patients (Case #4 and Case #9), authors relied only on public information revealed during court proceedings.

Disclosure (Authors): Triet Tran, Joan Steffen, Kate Clancy, Muna Yimam and Tess Bird work for Dr. David Egilman, who served as an expert witness in litigation at the request of people who were injured as the result of using talcum powders. Mr. Tran, Ms. Steffen, Ms. Clancy, Ms. Yimam and Dr. Bird were not compensated by law firms for work on this paper and the lawyers for the injured plaintiffs did not review this paper and had no input into the content of the paper.

Dr. David Egilman reports payments from lawyers related to the submitted work. Dr. David Egilman served as an expert witness in litigation at the request of people who were injured as the result of using talcum powders; plaintiffs' lawyers paid for the patient exams taken by Dr. Egilman as part of his expert witness work. He has also served as an expert witness at the request of companies who have been sued for exposure to asbestos from their mines or products. He was not compensated for work on this paper and the lawyers for the injured plaintiffs did not review this paper and had no input into the content of the paper.

Dr. Mark Rigler and Dr. William Longo report payments from lawyers related to the submitted work. Both served as an expert witness in litigation at the request of people who were injured as the result of using talcum powders; they originally performed the tissue analysis for talc and asbestos as part of their expert witness work and were paid by plaintiffs' lawyers for their work. They were not compensated for work on this paper and the lawyers for the injured plaintiffs did not review this paper and had no input into the content of the paper.

Disclaimer: Historic testing of talc for asbestos is limited in methodology and scope. Courts and plaintiff lawyers have agreed, without the knowledge or permission of their clients, to keep secret some of the documents reported here; these documents became public during court proceedings over the objections of J&J and Imerys. Many documents remain sealed.

ABSTRACT

Objective:

Asbestos is a known cause of ovarian cancer. We report 10 cases of serous ovarian cancer among users of Johnson & Johnson (J&J) asbestos-containing "cosmetic" talc products.

Methods:

We conducted an asbestos exposure assessment during talc application and analyzed surgical tissues and talc containers for asbestos and talc.

Results:

Talc was found in all cases and tremolite and/or anthophyllite asbestos was found in 8/10 cases. The asbestos fibers found in the "cosmetic" talc containers matched those found in tissues. We estimated inhaled asbestos dose ranged from 0.38 to 5.18 fiber years.

Conclusion:

We provide evidence that the inhaled dose of asbestos/fibrous talc from "cosmetic" talc use causes ovarian cancer. The unique combination of the types of asbestiform minerals detected in cancerous tissue and "cosmetic" talc is a fingerprint for exposure to asbestos-containing talc.

KEYWORDS

Ovarian Cancer; Asbestos; Talc; Baby Powder; Cosmetics; Johnson & Johnson

INTRODUCTION

Known amongst oncologists as a "silent killer," ovarian cancer is the leading cause of death from all gynecologic cancers and the fifth leading cause of cancer-related deaths among women in the United States.[1] The American Cancer Society estimates that about 22,000 American women will be diagnosed and 13,850 will die of the disease in 2019.[2] In 2010, the agency determined that perineal talc powder use is possibly carcinogenic to humans (group 2b).[3]

Epidemiological studies have examined the relationship between perineal talc use and ovarian cancer. In a 1982 case-control study, Cramer et al. first reported an association between genital talc use and ovarian cancer.[4] At least 32 subsequent epidemiologic studies have examined the association between talc powder use and ovarian cancer.[5-36] High-grade serous carcinoma (HGSC) is the most common form of ovarian cancer and the type of ovarian cancer that has been most consistently associated with perineal use of cosmetic talc products.[6-8,10,12,14,15,24,27,29,32,33,36,37] Meta-analyses have consistently shown an increased risk of HGSC of about 1.3 for perineal talc use.[18,38-40]

Asbestos exposure by inhalation occurs during cosmetic talc use. [41,42] IARC concluded in 2009 that asbestos was a group 1 ovarian carcinogen.[43,44] Dr. H. Wyers' first reported a case of ovarian cancer in a woman with asbestosis in 1949. [45] Twenty-seven epidemiologic studies have since examined the relationship between asbestos exposure and ovarian cancer.[46-72] Nine of these 27 studies report a statistically significant elevation in ovarian cancer risk.[46-48,51,61,62,68,69,71] Epidemiologic findings have demonstrated consistency in different populations: studies of asbestos and ovarian cancer have shown a statistically-significant association among women in different countries with exposures to different types of asbestos fibers and in various occupational and environmental settings.[46-48,51,61,62,68,69,71] Epidemiologic research also suggests a dose-response relationship for asbestos and ovarian cancer when comparing low-exposure and high-exposure subgroups.[47,72] Camargo et al. (2011) performed a meta-analysis of 18 cohort studies of occupational asbestos exposure and reported a pooled standardized mortality ratio (SMR) for ovarian cancer of 1.77 (95% CI, 1.37-2.28).[73]

Epidemiologic studies of talc and ovarian cancer have generally accepted representations by talc mining and manufacturing companies that consumer talc has been asbestos-free since 1976.[6-8,10,12,14,15,24,25,27,29,32,36] However, studies show that consumer talc contains asbestos and a review of the world's largest talc producers records indicated that talc mines contained asbestos, that asbestos cannot be removed from talc, and that talc used in cosmetics was not asbestos-free.[41,74-82] Case control and cohort studies of talc use and ovarian cancer have not differentiated inhalation and perineal talc exposures, and have not considered inhalation exposures in their analyses; this has contributed to misclassification of

exposed cases and inaccurate dose-response assessments.[42] In addition, industry marketing studies from the 1970s indicate that up to 85% of women used talc powders thus many "controls" were probably exposed to asbestos containing talcs. [42,83]

We report ten cases of serous ovarian cancer among users of asbestos-containing J&J cosmetic talc products. Unlike most previous studies on talc and ovarian cancer, we focused on inhalation exposures to asbestos during various talc uses and not perineal exposure. [4,6,12,40] We measured inhalation exposures during perineal application of asbestos-containing cosmetic talc. Based on exposure histories, we estimate the dose of inhaled asbestos and the increase in ovarian cancer risk for each case. Our case series also includes tissue analysis for talc and asbestos in both product and cancer tissue. By synthesizing current knowledge of asbestos carcinogenicity and evidence of asbestos in consumer talc products, our case series provides novel insight into the link between cosmetic talc use and ovarian cancer.

MATERIALS AND METHODS

We report ten cases of serous ovarian cancer in women who primarily or exclusively used a variety of Johnson & Johnson (J&J) cosmetic talc products including Johnson's Baby Powder (JBP), Shower to Shower (STS), and STS Shimmer. [84]These cases were identified among a group of 22 plaintiffs in Ingham et al. vs. Johnson & Johnson et al. All plaintiffs were diagnosed with ovarian cancer after exposure to J&J cosmetic talc products and TEM tissue analysis for talc and asbestos was performed for 10 of these plaintiffs. We only report on the ten plaintiffs for whom TEM tissue analysis was completed.

There was no requirement for ethics review or institutional review board approval because this research was not experimental and patients participated voluntarily in conjunction with a lawsuit. Informed consent for publication was obtained from all living patients. One patient (Case #8) passed away after her exposure history was collected but before consent for publication was obtained. In this case, consent was obtained from the surviving spouse. For the remaining 2 deceased patients (Case #4 and Case #9), authors relied only on public information revealed during court proceedings. For the exposure assessment, the researcher wore a respirator and was decontaminated post-assessment. The researcher was not exposed to any risk, required to reveal personal information or subjected to specimen collection. The assessment did not meet the requirements to necessitate IRB approval. [85]

PATIENT HISTORIES

Medical histories, exposure histories (history questionnaire attached as Appendix 1, http://links.lww.com/JOM/A685), and physical examinations were collected for all living patients (8/10 cases). Exposure histories included questions about talc powder use and other sources of asbestos

exposure. We analyzed the frequency and duration of talc uses for each case. For the two deceased patients (Case #4 and Case #9), a rough exposure history was compiled from the testimony of relatives who were familiar with each patient. Available medical records were also reviewed for all cases.

EXPOSURE ASSESSMENT- PERINEAL APPLICATION

The exposure assessment was completed in a 15'x15'x8' room with appropriate negative asbestos airflow technology. The experiment was videotaped using two Sony Model HDR-CX900 cameras with alternating Tyndall and standard lighting. (See Appendix 2, http://links.lww.com/JOM/A686.) Area and background samples were collected using four high-volume area sampling pump stations set up 5' to 6' from the talc user; these pump stations used 25mm air cassettes containing 0.8µm pore size mixed cellulose ester (MCE) filters with 5.0µm backing pads and were calibrated to run at 10 liters/minute. Personal samples were collected using four low-volume pumps affixed to the talc user with the cassettes adjusted to be in the breathing zone of the investigator; the "personal" pumps were calibrated to 2.5 liters/minute. During the experiment, air samples were collected for 5 minutes from all sources.

A researcher wearing personal protective equipment and "personal" air pumps used a metal container of JBP for the experiment. Based on JBP advertisements featuring product images, we estimated that the JBP used in this test had been manufactured sometime in the 1950s and sourced from the Val Chisone mine. [86,87](See Appendix 3, http://links.lww.com/JOM/A688 for images of JBP product tested and for full written report on exposure assessment.) J&J used this mine source from 1946 until 1968 and 1980-1981. [86-88] From 1969 to 2003, J&J used Vermont talc in their powder products and later switched to Chinese. [42,89] Using t-test analysis, the asbestos content (fibers per gram) in all the bottles tested were statistically comparable across these three talc sources. (See Appendix 4, http://links.lww.com/JOM/A689)

The JBP can was weighed before the experiment using a Fisher Scientific balance. The researcher wore a bikini bottom over an inner pair of boxer briefs and sat on a chair in the middle of the room for the experiment. To simulate perineal talc application, the researcher shook the talc powder into his hand twice and then rubber the powder into the upper leg area. This was repeated for the other leg. Then, the researcher stood, pulled the bikini bottom down and away from the body, and applied 2 squeezes of talc powder into the bikini bottom. The researcher released the briefs into place and sat down on the chair for the remainder of the study. The metal container of JBP was weighed again following the study. After the study, 2 field blanks were opened inside the study room.

A total of four background samples, four personal samples, and four area samples were collected along with two field blanks. All twelve air samples were analyzed for asbestos by the NIOSH 7400 PCM

method using "A" counting rules and by the NIOSH 7402 TEM method.[90,91] For TEM analysis, amphibole asbestos fibers or bundles with substantially parallel sides and an aspect ratio of 3:1 or greater, at least longer than 5.0 μm in length and greater than 0.25 μm were counted as per NIOSH 7402 asbestos structure sizing rules.[91] The four personal air samples were also analyzed by the NIOSH 7402 method for fibrous talc particles.[91] The two field blanks were analyzed for asbestos by PCM and TEM in accordance with NIOSH 7400 and NIOSH 7402.[90,91]

DOSE CALCULATIONS

For each case, we calculated asbestos dose in environmental fiber years (for consistency with the EPA risk assessment model) and in total fibers inhaled (to account for changes in respiratory intake in infancy vs. adulthood).[92] We used the asbestos dose in environmental fiber years to calculate the excess risk. (See section on Dose-Response Risk Assessment.)

We calculated total asbestos dose based on the four most common usages of J&J talc powder reported among the ten cases: perineal application (10/10), upper body powdering (9/10), exposure as an adult during diapering (8/10) and exposures as an infant during diapering (7/10). For each of these scenarios, we incorporated the intensity of the exposure (f/cc), duration of each exposure (minutes) and total number of applications (from exposure histories) to calculate the dose. Although we did not adjust for latency, we excluded exposures that occurred after ovarian cancer diagnosis. Fibrous talc exposures from powdering was excluded from our calculations except exposure from baby diapering.[41] Dement et al. (1972) did not differentiate type of fiber detected. [93]

For perineal powdering exposures, we relied on measurements from our exposure assessment. (See above.) Air samples were collected over the course of 5 minutes in this test.

For upper body powdering, we used Gordon et al.'s (2014) measurements for shaker application of cosmetic talc powder to the underarm, shoulder, and upper arm area.[41] Gordon et al. (2014) used Cashmere Bouquet, which used the same Italian mine source as J&J (Val Chisone) from 1940 until 1992. [94,95] Gordon et al. (2014) found that users were exposed to 1.9 f/cc of asbestos fibers over the course of 5 minutes.[41]

For exposures during diapering, Dement et al. (1972) from NIOSH found that an adult is exposed to 2.2 f/cc and that a baby is exposed to 1.8 f/cc over the course of two minutes.[93] When subjects reported that their parents had used talc on them during diaper changes as an infant, we relied on diaper changing norms to estimate infant exposures. US market research and survey data shows that diaper changes typically occur 8-10 times per day for infants (0-6 months) and 4-6 times per day for toddlers (6-24

months).[96-98] Diaper changing frequency in the US also changed over time: the average number of diaper changes per day over the first 2 years of life dropped from eight times per day in the 1960s to 5-6 times per day by the 1980s due to improvements in disposable diapers and reduction in cloth diaper use.[97,99] Since all of the women in our series were born prior to 1975, we assumed that diaper changes occurred eight times per day for two years.

We calculated the dose for each case in fiber years $\left(\frac{f}{cc} \bullet year\right)$ using the same conversions as Anderson et al (2017).[100] For consistency with the EPA dose-response curve used for our risk assessment, we calculated the total duration of exposure based on a continuous, 24-hour exposure period (525,600 min/year) until date of diagnosis.[92]

Formula 1

We also calculated the total number of asbestos fibers inhaled in each case. For adults, we used the National Research Council (NRC)'s estimate of "an annual inhaled air volume of 7,300 m^3" and formula to convert the dose from fiber years to total fibers.[101] We relied on measurements of infant lung volume from Hall (1955) and on median infant respiratory rates calculated by Fleming et al. (2011) to estimate the total inhaled air volume for infants from age 0 to 2.[102,103] Using time-weighted averages for tidal volume and respiratory rate, we calculated that infants breathed 11,025,072,000 ccs in the first 2 years of life, or 5,512,536,000 ccs per year on average.

Formula 2 & 3:

We added together adult and infant exposures to calculate the exposures in total number of asbestos fibers. See Appendix 5, http://links.lww.com/JOM/A690 for the full dose calculations for each case.

Dose-Response Risk Assessment

We developed a method to apply the EPA dose-response curves for inhaled asbestos and mesothelioma risk to ovarian cancer risk. [92] First, we examined the EPA dose-response table for mesothelioma from environmental asbestos exposure (24-hours, 365 days per year). [92] Utilizing the EPA dose-response estimates, we extrapolated a formula for the line of best fit for mesothelioma risk.

We then identified studies that reported mesothelioma and ovarian cancer rates in the same cohort and calculated comparative risk of mesothelioma versus ovarian cancer for each study.[58,62,63,68,71] (See Table 1.)

Using these studies, we calculated the geometric mean comparative risk of contracting mesothelioma versus ovarian cancer from the same asbestos exposures. We applied this comparative risk to the line of best fit for mesothelioma based on the EPA dose-response data to determine a formula for risk of ovarian cancer.

The subjects of the EPA occupational exposure study were entirely men.[92] Since women are more susceptible to cancer from asbestos exposure, we used Lacourt's (2014) findings comparing the mesothelioma odds ratio (OR) in men versus women with the same exposures to adjust the formula for the increase in cancer risk for women.[104] At total doses >0-0.1 fiber years, women were 1.725 times more likely to have mesothelioma than men.[104] At total doses >0.1-1 fiber years, women were 2.855 times more likely to have mesothelioma than men.[104] We applied these ratios to the EPA dose curve calculated to obtain a better estimate of the ovarian cancer dose-response in women.

The resulting dose-response curve for inhaled asbestos and ovarian cancer is shown in Figure 1. We used each case's asbestos dose estimate in fiber years to identify their relative lifetime risk of developing ovarian cancer along the dose-response curve. We then compared each case's risk of contracting ovarian cancer due to inhaled asbestos exposure to the expected incidence of ovarian cancer for those without asbestos exposure: 11.4 per 100,000 from the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) Program.[105]

TISSUE ANALYSIS FOR ASBESTOS AND TALC

Samples from a combination of the left and right ovaries, left and right fallopian tubes, and left and right pelvic lymph nodes were obtained from the hospital for each of the ten patients. Tissues were analyzed to identify and quantify talc and asbestos content in the tissue.

For tissue analysis, a small portion of the tissue in each block was removed with a clean razor blade and placed in a pre-weighed 20-30 mL borosilicate glass vial. The vial was filled with ten mL of filtered extraction solvent (hexane) and placed in a 60°C water bath. The filtered extraction solvent was replaced every twenty minutes for a total of three changes. After the last extraction solvent change, two changes of filtered ethanol (10 mL, each) ten minutes each were performed, then the tissue piece(s) were dried at 110 – 120°C.

Tissue samples were digested with 15-30 mL of filtered sodium hypochlorite (appx. 8.0% bleach). After digestion, the remaining digested material was filtered through a 25 mm, 0.4 micron polycarbonate (PC) filter. The filter containing the tissue residue was dried and subsequently prepared for TEM examination.

A paraffin control sample (wax blank) was obtained by dissolving a known quantity of the paraffin blocks (devoid of tissue) in ten mL of filtered extraction solvent and the dissolved solvent/wax solution was then filtered onto a twenty-five mm, 0.4 micron PC filter. The filter was allowed to dry and then prepared for TEM analysis. A process blank (sample vial) was prepared in the same manner and followed the wax blank and tissue sample vials through all steps.

For TEM analysis, 100 - 300 grid openings were analyzed for all asbestos and talc structures at a magnification of between 4,000 and 20,000X. As per standard TEM analysis protocols, asbestos fiber/bundle identification was done by morphology (substantially parallel sides and length to width ratio of at least 5:1), length (greater than 0.5 µm in length), selected area electron diffraction (SAED), and energy dispersive X-ray spectroscopy (EDS).[106-112] Talc structures (platy and fibrous) were identified morphologically, by selected area diffraction (SAED), and energy dispersive spectroscopy (EDS).

RESULTS

EXPOSURE ASSESSMENT

Total weight used during the application process was 4.05g of talc powder. For the 5 minute sampling time, the average total fiber exposure was 4.52 f/cc (5.86, 4.38, 3.85, and 3.98 f/cc), the average asbestos exposure was 2.57 f/cc (4.51, 1.88, 2.07, and 1.81 f/cc) and the average talc exposure was 1.95 f/cc (1.35, 2.50, 1.78, and 2.16 f/cc) for the talc user personal samples. For area samples, the average total fiber exposure was 0.41 f/cc (0.52, 0.28, 0.42, 0.40 f/cc), the average asbestos exposure was 0.2 f/cc (0.31, 0.20, 0.13, and 0.16 f/cc) and the average fibrous talc exposure was 0.19 f/cc (0.13, 0.08, 0.29, and 0.24 f/cc). The type of asbestos fiber identified in all samples was tremolite asbestos. No fibers were detected in the background samples or field blanks. The complete exposure assessment report, including count sheets and fiber images, is available as Appendix 3, http://links.lww.com/JOM/A688.

DOSE CALCULATIONS AND RISK ASSESSMENT

Results for dose calculations, risk assessment and tissue analysis are summarized in Table 2. See Appendix 5, http://links.lww.com/JOM/A690 for complete past medical history, history of present illness, other ovarian risk factors, exposure history, and dose calculations for each case.

STS was comprised of talcum powder mixed with cornstarch. The STS products contained between 80% to 100% talc sourced from the same mines as JBP.[84] Only 4 cases used these products for brief or unknown periods of time. Case #3 reported infrequent use of unidentified facial make-up powder, and Case #6 reported infrequent use of generic store-brand talcum powder. We could not calculate exposures for the brief use of these unknown products.

All cases had pathologically confirmed serous ovarian cancer. Age at diagnosis ranged from 41 to 78 years, with a mean age at diagnosis of 51.1 years and median age at diagnosis of 50 years. By contrast, the median age of ovarian cancer diagnosis in the United States is 63 with most cases occurring in women aged 55 to 64. Seven of 10 cases tested negative for BRCA mutations; 2 cases were never tested (#2 and #5) and one case (#8) tested positive for BRCA2 variant L771V.

All cases reported perineal talc application; the frequency of perineal powdering with talc ranged from once per day to ten times per day and the duration ranged from 24 years to 47 years. Nine of ten cases reported upper body powdering with talc ranging from 1 to 5 times per day and lasting from 20 to 47 years. Seven of 10 cases reported that their parents used talc powder on them during diaper changes and eight of 10 cases used talc powder during diapering. The total asbestos dose from talc powder use ranged from 2,774,000,000 to 37,742,501,440 asbestos fibers (0.38-5.18 fiber years) and the average dose was 9,308,551,008 asbestos fibers (1.28 fiber years). No other known asbestos exposure was identified for any of the cases. Based on EPA dose dose-response estimates, the risk of developing ovarian cancer due to inhaled asbestos exposure was calculated to be 2.3 to 31.1 times greater in these cases compared to baseline risk for ovarian.[105] On average, the risk of ovarian cancer increased 7.7-fold among these cases.

TISSUE ANALYSIS

Talc and/or asbestos was identified in the tissue from all cases. Platy talc was found in 9/10 cases (90%) with an average concentration of 264,487 structures per gram (s/g) (range: 0 – 2,057,640 s/g). Fibrous talc was found in 8/10 cases (80%) with an average concentration of 5,878 s/g (range: 0 – 21,545 s/g). Tremolite asbestos was found in 6/10 cases (60%) with an average concentration of 6,488 s/g (range: 0 – 22,000 s/g). Anthophyllite asbestos was found in 4/10 cases (40%) with an average concentration of 2,393 s/g (range: 0 – 12,000 s/g). Ferro-anthophyllite asbestos was also identified in 2 cases (20%), winchite and richterite asbestos were identified in 1 case (10%), and crocidolite asbestos was identified in one case (10%). Two tremolite structures with aspect ratios less than 5:1 were observed in one case, but were not counted as asbestos.

In the 'possible fallopian tube B' tissue of case #2, a cluster measuring 20.0 x 16.0 µm was identified composed of 36 counted talc plates, two fibrous talc structures and one tremolite fiber. (See Figure 2.)

DISCUSSION

This case series identified asbestos and/or talc in the tissue of ten women diagnosed with serous ovarian cancer and exposed to J&J cosmetic talc products. Prior to their ovarian cancer diagnosis, these women were exposed to as much as 2,774,000,000 to 37,742,501,440 asbestos fibers (0.38-5.18 fiber years) due to their use of J&J cosmetic talc products. In all reported cases, asbestos exposures due to J&J talc use resulted in a substantial increase in ovarian cancer risk (2.3-31.1) based on our model. Early median age of diagnosis (50 in this case series versus 63 nationally), and the EPA dose response table, indicates that asbestos exposure in infancy may cause ovarian cancer to occur sooner than it would have occurred absent this exposure. [92] .[105]

The asbestos type found in the perineal talc use inhalation exposure assessment (tremolite asbestos) and the predominant asbestos types identified in these tissue samples (tremolite and anthophyllite asbestos) matched the fiber types previously identified in cosmetic talc products and in talc mines.[41,74,75,77-81] (See Table 3.) Researchers have previously identified anthophyllite asbestos in Johnson's Baby Powder (by TEM analysis),[79] amphibole needles and fibers in baby powder sourced from Vermont,[76,77] and tremolite asbestos fibers in commercial talc produced prior to 1975 from J&J's talc source in Val Chisone, Italy.[81,89]

In 2017, a bundle of tremolite asbestos fibers was found in a bottle of JBP purchased by Case #3 in 2014. (See Appendix 6, http://links.lww.com/JOM/A691 for full purchase report.) Tremolite asbestos was also identified in Case #3's right pelvic lymph node. (See Figure 3.) Winchite and richterite asbestos were found in the tissue in 1 case. However, richterite was called sodium tremolite prior to 1978.[113] Winchite is found in talc from the Allamoore, Texas mine and may have contaminated J&J Italian talc processed at the same plant in the 1970s.[114-118] Similarly, Transite pipes present in Royston Plant may have contaminated J&J talc with crocidolite.[119] [120] Furthermore, Colgate acknowledges that there is crocidolite in some talc.[121]

The most common structures identified by tissue analysis (platy talc, fibrous talc, tremolite and anthophyllite asbestos) strongly indicate talc powder as the source of asbestos exposure in these cases. Tremolite asbestos has had minor commercial production in India and Italy and is mainly found as an accessory mineral in talc, vermiculite, and chrysotile.[122-124] Anthophyllite asbestos, which occurs as an accessory mineral in talc and chrysotile, has also had limited commercial use.[123-125] Anthophyllite and tremolite together account for less than 1% of asbestos production and consumption worldwide.[124]

None of the cases reported in this series had any known history of alternative asbestos or vermiculite exposure and no chrysotile or vermiculite was found in any of the tissue samples. Churg and

Warnock (1979) performed a population study of lung asbestos and noted that "… in women a major source [of asbestos fibers] may be cosmetic talc, which is often contaminated with anthophyllite and tremolite."[126] Finkelstein's (2018) analysis of mesothelial tissue found a statistically significant association for tremolite detected with talc in tissue.[130] This association was higher for females, 82% of whom had talc in their tissue compared to 68% of males. [130] The increased use of talcum-based cosmetics by females, and the similar fiber type combination is a fingerprint of cosmetic talc migrating to the peritoneum. The combination of talc with tremolite and/or anthophyllite asbestos, as identified by Finkelstein (2018) and the ten cases reported here, are a fingerprint for exposure to asbestos-containing talc. [127-129] (Appendix 7, http://links.lww.com/JOM/A692: a chart of fibers detected in J&J compared with fibers in tissue). These results indicate that perineal use can result in important inhalation exposure to asbestos, which is accepted route of transmigration to the peritoneum and ovary.[131]

Our exposure assessment found that cosmetic talc users can be exposed to 2.57 f/cc asbestos in the breathing zone during perineal talc application; this finding was generally in agreement with previous studies of asbestos exposures during talc use. [41,93] The bottle of JBP used in this exposure assessment was tested by TEM which detected 15 million fibers per gram. Further analysis found asbestos in 56/90 JBP bottles with a range of 4,400 to 15,100,000 asbestos fibers per gram (appendix 4, http://links.lww.com/JOM/A689). For comparison, Gordon et al. (2014) conducted exam on 50 samples of a single brand of cosmetic talc, sourced from either Montana, North Carolina or Val Chisone. Gordon et al. (2014) found a range of 1,840 to 200 million asbestos fibers per gram.[41] Asbestos is not evenly distributed in talc ores and sampling cannot be completely representative of exposure. [88,132]

Gordon et al. (2014) selected a bottle with 18 million asbestos fibers per gram for the inhalation study. The results Gordon et al.'s (2014) simulation for body powdering 1.9 f/cc, comparable to our assessment of 2.57 f/cc asbestos exposure per application. Application of cosmetic talc varies greatly, including differences in product, application time, grams per use and location of application. In addition, talc is mined and milled prior to sale, potentially modifying fiber size or dispersing asbestos unequally in finished product. [133] Talc was sourced from various mines and processing methods changed over time, adding to the variability of asbestos content in talc-containing cosmetic products. However, our findings of an asbestos fingerprint in the tissue reveal that regardless of the dose, exposure to talc-containing cosmetic products is sufficient to cause ovarian cancer.

We relied on NIOSH measurements by Dement et al. (1972) to calculate exposures during diapering, however these measurements did not account for airborne asbestos exposures that continued after the sampling time.[93] Dement et al. (1972) collected air samples for two minutes during a simulated

diaper change with JBP, but another experiment in the same study indicated that exposures continued for at least three minutes and likely persisted for even longer.[93] Dement et al. (1972) used phase contrast microscopy and did not differentiate between asbestos and fibrous talc. [93] However, in 1968, NIOSH injected asbestos containing "cosmetic" talc into hamsters and detected tremolite asbestos bodies but no fibrous talc in the animal lungs.[134] Anderson et al. (2017) reported much lower levels during body dusting with talc (0 to 0.0039 f/cc). However, the microscopist in the Anderson et al. study originally identified 4 anthophyllite asbestos fibers in the air samples by TEM, but changed the result to transition fibers at the request of the project supervisor due to concern that the results would be used in litigation.[100,135]

Both our study and Gordon et al.'s (2014) exposures assessment used less talc powder than the average user: these experiments used 4.05 and 0.37 grams of talc respectively, but J&J's unpublished studies found that women used 8.16 grams and men used 13.02 grams of talc powder on average during body powdering.[41,136] Anderson et al. (2017) reported that subjects used 11.6 grams of talc on average to powder their bodies after showering.[100] Therefore, our use estimates were 3 to 20 times lower than Anderson et al. (2017) and J&J's.

We also excluded many reported talc uses from our dose calculations due to a lack of exposure data. For instance, three cases (#1, #3, and #5) regularly used talc powder on their sheets and pillows; several other cases also reported seeing and smelling dust in the air while cleaning the room where they regularly applied talc. (See Appendix 5, http://links.lww.com/JOM/A690 for complete exposure histories.) Although our findings indicate that asbestos is present in consumer talc products at a level sufficient to cause disease, our dose estimates may under or over estimate the total exposure to carcinogens in talc in these cases.

Burns et al. (2019) created a dose estimation-model for cosmetic talc, relying on previous assessments to predict asbestos exposure, including Moon et al. (2011), Gordon et al. (2014), Russell et al. (1979), and Anderson et. al. (2017). [41,100,136-138]Burns et al.'s (2019) assessment was based on an assumption of .1% level of asbestos in talc mathematical model that incorrectly reduced the exposure estimate by 1000. [137] For example, Gordon et al. (2014) reported, 4.8 f/cc, however, Burns et al.'s (2019) math model reduces this figure to 0.0048 f/cc. [41,137] In comparison, Addison et al. (1988) reported that dusts containing 0.1% asbestos may release 1.17-2.79 asbestos fibers/cc into the air, consistent with our measurements.[139]

Our tissue analysis results were consistent with previous reports of asbestos and/or talc in ovarian tissue.[136,140-144] (See Table 4.) The number of asbestos structures per gram, however, were approximately one order of magnitude lower in our study than in previous quantitative studies of asbestos in ovarian

tissue.[143] This discrepancy may be due to differences in tissue preparation and analytical procedures. Other quantitative studies relied on wet tissue weight for their analysis whereas we used a dry weight procedure.[143] Additionally, we counted 100-300 grid openings in our study while other studies appear to have counted the entire grid area.[143] We also found that some tissue samples contained "hot spots" with very high concentrations of asbestos and/or talc compared to the surrounding tissue. (See Figure 2.) The occurrence or absence of "hot spots" may also account for variability in reported asbestos concentrations in tissue. The predominant types of asbestos identified in our series (tremolite and anthophyllite asbestos) are the same as those most commonly reported in past studies.[140,143,144]

We did not consider latency in our risk estimate because our calculations followed the EPA risk assessment, which did not consider latency.[92] In addition, Pira et al. found that for asbestos-caused ovarian cancer "…the SMRs increased monotonically with time since first employment, although the number of deaths was small in several categories..."[68] Our omission of latency from this study is to remain consistent with the EPA assessment and reflect the lack of effect demonstrated by Pira et al.'s analysis.

We omitted fibrous talc from our risk assessment due to a lack of dose-response data in the published literature. IARC has previously classified fibrous talc as a Group 1 carcinogen and OSHA regulates fibrous talc per the asbestos standard.[3,43,145-147] Further research on the relationship between talc powder use and ovarian cancer should include studies of fibrous talc toxicity.

CONCLUSION

Of the ten reported cases of serous ovarian cancer, all were found to have talc and 8 were found to have asbestos in their tissue samples. The main types of asbestos identified in tissue, tremolite and anthophyllite, constitute a fingerprint for talc containing asbestos and indicate that "cosmetic" talc powder as the source of asbestos exposure in these cases. IARC has concluded that asbestos is an ovarian carcinogen.[43] IARC has likewise classified talc containing asbestiform fibers (including both asbestos and fibrous talc) as a carcinogen.[3,43,148] These cases provide more evidence of the causal link between asbestos, talc, and ovarian cancer and indicate that asbestos is present in consumer talc products at a level sufficient to cause disease.

.In 1973, J&J told the FDA that "Johnson & Johnson's policy of full cooperation with FDA and that if the results of any scientific studies show any question of safety of talc, Johnson & Johnson will not hesitate to take it off the market" and their corporate position is that there is no known safe level of exposure to asbestos.[149] J&J's studies have shown that asbestos has been present in its cosmetic talc ores since the 1950s. In 2019, the FDA has found asbestos in JBP sourced from Vermont

and China and Claire's cosmetics .[150,151] At least three retailers of cosmetic talc accept the causal relationship between talc use and ovarian cancer: Angel of Mine, Perfect Purity, and Assured Body and Foot Powders warn that "frequent application of talcum powder in the female genital area may increase the risk of ovarian cancer."[152] In addition, J&J's talc supplier Rio Tinto Minerals has warned its customers since 2006 of this risk in Material Safety Data Sheets (MSDS) for talc: "perineal use of talc-based body powder is possibly carcinogenic to humans."[153,154] J&J removes this warning from its talc MSDS and cosmetic talc products.[155] Because talc powder is a cosmetic product with no medical benefit, these warnings still do not warrant the sale of a products when the benefits cannot outweigh the risks, especially when there is a safer substitute.[156] [157,158]

J&J should comply with its self-proclaimed obligation to take talc-containing cosmetic products off the market "if the results of any scientific studies show any question of safety of talc, Johnson & Johnson will not hesitate to take it off the market." [149]

SUPPORTING INFORMATION

Appendix 1: Exposure history questionnaire

Appendix 2: Perineal exposure assessment video

Appendix 3: Full report of perineal exposure assessment

Appendix 4: Analysis of historical samples of JBP

Appendix 5: Detail on reported cases

Appendix 6: Report on analysis of JBP purchased by case #3

Appendix 7: Fiber Comparison chart

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FIGURE LEGENDS

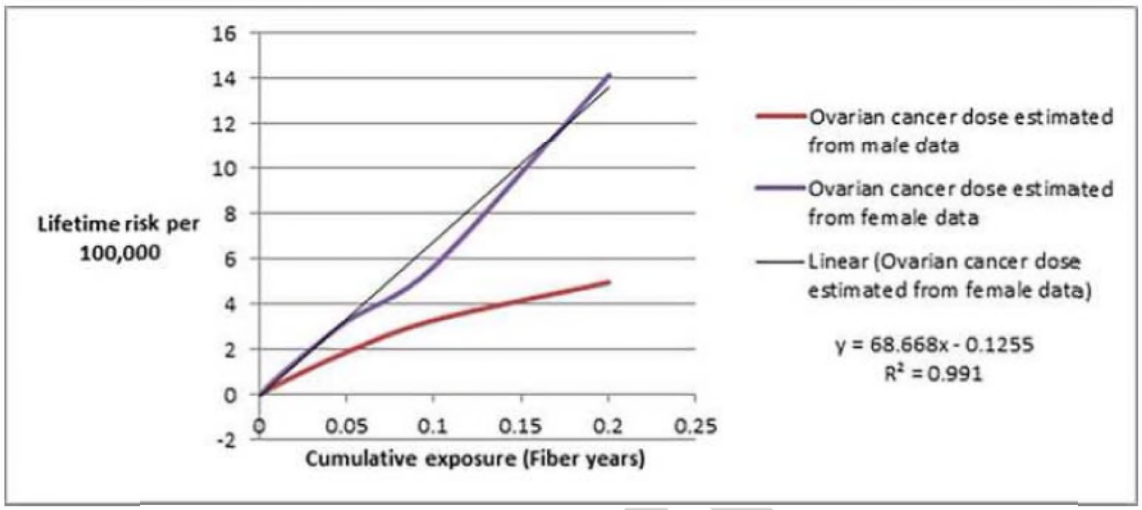

Figure 1: Ovarian cancer dose response (adjusted for difference in female mesothelioma risk)

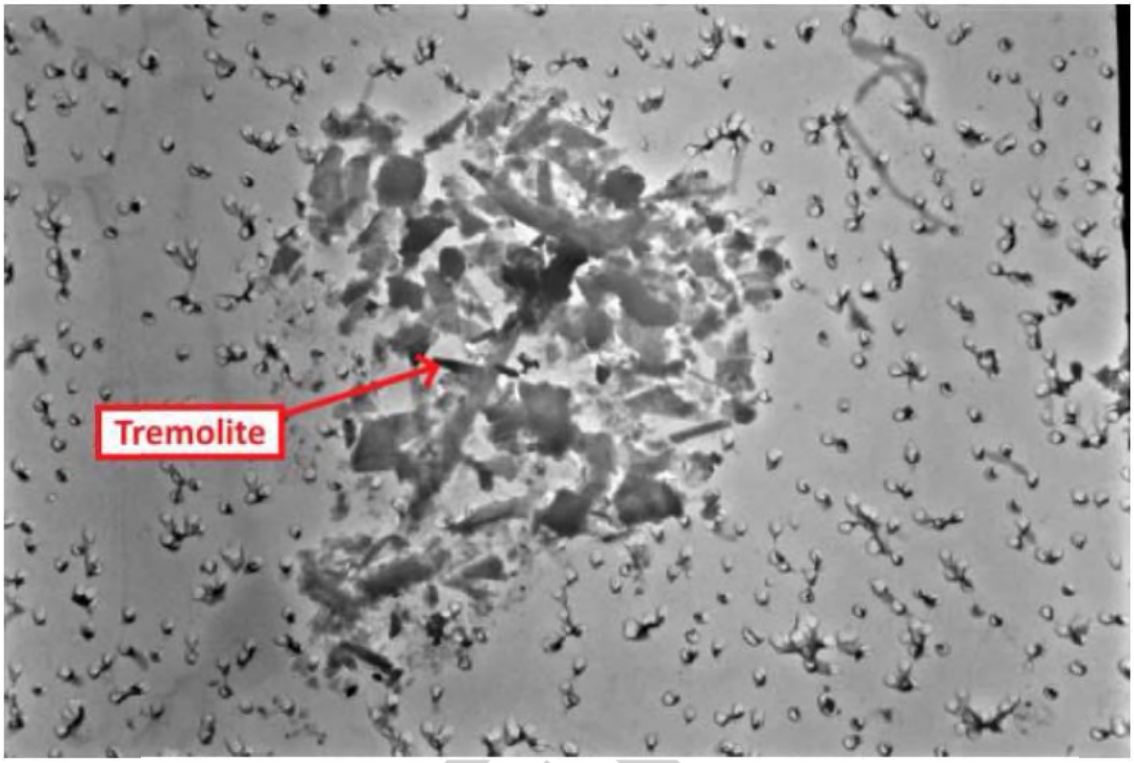

Figure 2: TEM image of cluster measuring 20.0 x 16.0 um composed of 36 counted talc plates, 2 fibrous talc structures and 1 tremolite fiber identified in "possible fallopian tube B" tissue of case #2.

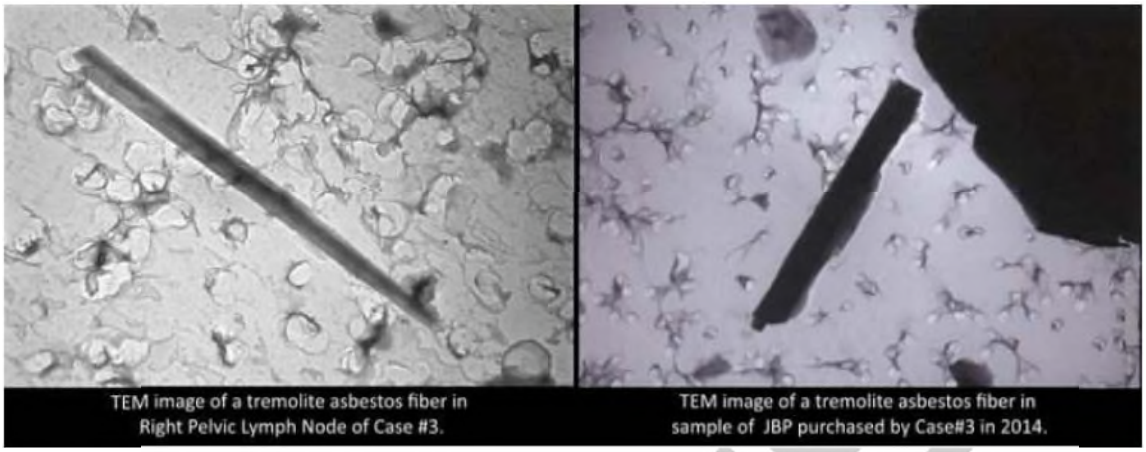

Figure 3: TEM images of a tremolite asbestos fibers in Case #3 right pelvic lymph node tissue (left) and in sample of JBP purchased by Case#3 in 2014 (right).

Table 1: Studies with both mesothelioma and ovarian cancer rates in the same cohort and calculated comparative risk of mesothelioma to ovarian cancer in female-only cohorts.

Study	Mesothelioma risk (RR)	Ovarian cancer risk (RR)	Comparative risk M/OC
Loomis 2009	10.92	1.23	8.88
Magnani 2008	51.49	2.27	22.68
Pira 2016	51.3	3.03	16.93
Wang 2013	166.67	7.69	21.67
Wilczyńska 2005	22.67	1.76	12.88
Geometric mean of comparative risk			15.69

Table 2: Summary of Cases

Case Number	***Diagnosis***	***Age at Diagnosis***	***Talc Exposure History***				***Calculated Asbestos Dose***	***Relative Increase in Ovarian Cancer Risk***	***Pathological Examination***	
			Perineal powdering	*Upper body powdering*	*Infant exposure during diapering*	*Adult exposure during diapering*			*Tissue examined*	*Findings (structures per gram of tissue)*
1	Metastatic high grade papillary serous carcinoma	45	10x/day, 40yrs	5x/day, 40yrs	8x/day, 2yrs	10x/day, 8yrs	37,742,501,440 fibers, (5.18 fiber years)	31.1	Ovary (R)	Platy talc (333 s/g), Fibrous talc (4,000 s/g), Ferro-anthophyllite (3,667 s/g)
									Ovary (L)	Fibrous talc (1,200 s/g), Ferro-anthophyllite (399 s/g)
									Fallopian tube (R)	NSD*
									Fallopian tube (L)	----†
									Pelvic Lymph Node (R)	----†
									Pelvic Lymph Node (L)	NSD*

Case Number	***Diagnosis***	***Age at Diagnosis***	***Talc Exposure History***				***Calculated Asbestos Dose***	***Relative Increase in Ovarian Cancer Risk***	***Pathological Examination***	
			Perineal powdering	*Upper body powdering*	*Infant exposure during diapering*	*Adult exposure during diapering*			*Tissue examined*	*Findings (structures per gram of tissue)*
2	Poorly differentiated high grade serous ovarian carcinoma	53	1x/day, 36yrs	1x/day, 23yrs	8x/day, 2yrs	7.5x/day, 7.5yrs	4,892,501,440 fibers, (0.68 fiber years)	4.1	Ovary A	NSD*
									Ovary B	Platy talc (323 s/g)
									Possible fallopian tube A	NSD*
									Possible fallopian tube B	Platy talc (56,700 s/g), Fibrous talc (4,720 s/g), Tremolite (22,000 s/g)
3	High grade serous carcinoma	49	3x/day, 39yrs	3x/day, 20yrs	8x/day, 2yrs	7x/day, 5yrs	11,535,501,440 fibers, (1.59 fiber years)	9.6	Ovary, fallopian tube (R)	Platy talc (2,001,503 s/g), Fibrous talc (13,343 s/g)
									Adnexa, fallopian tube (L)	Platy talc (12,308 s/g), Fibrous talc (8,202 s/g)

Case Number	***Diagnosis***	***Age at Diagnosis***	***Talc Exposure History***				***Calculated Asbestos Dose***	***Relative Increase in Ovarian Cancer Risk***	***Pathological Examination***	
			Perineal powdering	*Upper body powdering*	*Infant exposure during diapering*	*Adult exposure during diapering*			*Tissue examined*	*Findings (structures per gram of tissue)*
									Pelvic Lymph Node (R)	Tremolite (15,670 s/g), Winchite (15,670 s/g),‡ Richterite (15,670 s/g)‡
									Pelvic Lymph Node (L)	Platy talc (43,829 s/g)
4	Poorly differentiated serous adenocarcinoma	78	1x/day, 43yrs ¶	unknown¶	unknown¶	unknown¶	2,774,000,000 fibers, (0.38 fiber years)	2.3	Ovary (R)	Platy talc (2,860 s/g), Anthophyllite (952 s/g)
									Ovary (L)	Tremolite (604 s/g)
									Fallopian tube (R)	Platy talc (30,000 s/g)
									Fallopian tube (L)	Fibrous talc (868 s/g)
									Pelvic Lymph Node (R)	Platy talc (12,600 s/g)

Case Number	***Diagnosis***	***Age at Diagnosis***	***Talc Exposure History***				***Calculated Asbestos Dose***	***Relative Increase in Ovarian Cancer Risk***	***Pathological Examination***	
			Perineal powdering	*Upper body powdering*	*Infant exposure during diapering*	*Adult exposure during diapering*			*Tissue examined*	*Findings (structures per gram of tissue)*
									Pelvic Lymph Node (L)	Platy talc (17,600 s/g), Tremolite (2,510 s/g)
5	Low grade serous carcinoma	52	1x/day, 47yrs	1x/day, 47yrs	8x/day, 2yrs	10x/day, 10yrs	7,812,501,440 fibers, (1.08 fiber years)	6.5	Ovary (R)	Platy talc (10,900 s/g), Fibrous talc (1,810 s/g)
									Ovary (L)	Platy talc (25,000 s/g), Fibrous talc (5,000 s/g), Tremolite (5,000 s/g)
									Int. Iliac lymph node (R)	Platy talc (77,200 s/g), Fibrous talc (7,720 s/g), Tremolite (3,860 s/g), Anthophyllite (3,860 s/g)

Case Number	***Diagnosis***	***Age at Diagnosis***	***Talc Exposure History***				***Calculated Asbestos Dose***	***Relative Increase in Ovarian Cancer Risk***	***Pathological Examination***	
			Perineal powdering	*Upper body powdering*	*Infant exposure during diapering*	*Adult exposure during diapering*			*Tissue examined*	*Findings (structures per gram of tissue)*
									Comm. Iliac lymph node (R)	Platy talc (50,600 s/g)
6	High grade serous papillary carcinoma	51	1x/day, 40yrs	1x/day, 40yrs	8x/day, 2yrs	10x/day, 10yrs	7,009,501,440 fibers, (0.97 fiber years)	5.8	Adnexa, tumor/ovary (R)	Platy talc (21,300 s/g)
									Adnexa, tumor/ovary (L)	Platy talc (4,720 s/g)
									Adnexa, fallopian tube (R)	Platy talc (12,000 s/g), Tremolite (12,000 s/g), Anthophyllite (12,000 s/g)
									Adnexa, fallopian tube (L)	Platy talc (13,700 s/g)
									Pelvic Lymph Node (L)	Platy talc (11,500 s/g)

Case Number	***Diagnosis***	***Age at Diagnosis***	***Talc Exposure History***				***Calculated Asbestos Dose***	***Relative Increase in Ovarian Cancer Risk***	***Pathological Examination***	
			Perineal powdering	*Upper body powdering*	*Infant exposure during diapering*	*Adult exposure during diapering*			*Tissue examined*	*Findings (structures per gram of tissue)*
7	Serous adenocarcinoma	56	1x/day, 37yrs	1x/day, 37yrs	unknown	7.5x/day, 6yrs	5,183,000,000 fibers, (0.71 fiber years)	4.3	Ovary (R)	Platy talc (8,740 s/g), Fibrous talc (1,090 s/g)
									Ovary (L)	Platy talc (10,500 s/g)
									Fallopian tube (R)	Platy talc (8,500 s/g)
									Fallopian tube (L)	Platy talc (10,900 s/g)
8	High grade ovarian serous carcinoma	44	1x/day, 24yrs	1x/day, 24yrs	unknown	3.5x/day, 4yrs	2,993,000,000 fibers, (0.41 fiber years)	2.5	Ovary (R)	Platy talc (3,340 s/g), Ferro-anthophyllite (1,670 s/g), Crocidolite (1,670 s/g)
									Ovary (L)	Platy talc (799 s/g)

Case Number	***Diagnosis***	***Age at Diagnosis***	***Talc Exposure History***				***Calculated Asbestos Dose***	***Relative Increase in Ovarian Cancer Risk***	***Pathological Examination***	
			Perineal powdering	*Upper body powdering*	*Infant exposure during diapering*	*Adult exposure during diapering*			*Tissue examined*	*Findings (structures per gram of tissue)*
									Fallopian tube (R)	Platy talc (9,690 s/g), Fibrous talc (1,380 s/g), Tremolite (1,385 s/g), Anthophyllite (1,385 s/g)
									Fallopian tube (L)	Platy talc (7,400 s/g), Tremolite (1,850 s/g)
9	Poorly differentiated serous papillary adenocarcinoma§	41	1x/day, 42yrs¶	1x/day, 42yrs¶	8x/day, 2yrs¶	n/a¶	4,965,501,440 fibers, (0.69 fiber years)	4.1	Ovary (R)	NSD*
									Ovary (L)	NSD*
									Fallopian tube (R)	NSD*
									Fallopian tube (L)	NSD*
									Pelvic Lymph Node (L)	Fibrous talc (8,770 s/g)

Case Number	***Diagnosis***	***Age at Diagnosis***	***Talc Exposure History***				***Calculated Asbestos Dose***	***Relative Increase in Ovarian Cancer Risk***	***Pathological Examination***	
			Perineal powdering	*Upper body powdering*	*Infant exposure during diapering*	*Adult exposure during diapering*			*Tissue examined*	*Findings (structures per gram of tissue)*
10	High-grade ovarian papillary serous carcinoma	42	2x/day, 32yrs	2x/day, 32yrs	8x/day, 2yrs	8x/day, 4yrs	8,177,501,440 fibers, (1.13 fiber years)	6.8	Ovary, fallopian tube (R)	Platy talc (10,800 s/g)#
									Ovary, fallopian tube (L)	Platy talc (5,520 s/g)
									Pelvic Lymph Node (R)	Platy talc (79,300 s/g)
									Pelvic Lymph Node (L)	Platy talc (84,400 s/g)

Table 2 Legend:

* No asbestos or talc structures detected.

† Tissue received, but not analyzed.

‡ Winchite and richterite asbestos were considered tremolite prior to 1978.

¶ Patient deceased; exposure history based on recollections of family and friends.

§ The final pathology report also noted minor components of transitional cell and mucinous carcinoma.

2 tremolite structures were reported with an aspect ratio of less that 5:1 that were not counted.

Table 3: Summary of studies reporting asbestos in consumer talc products

Study	Test Method	Summary of Findings
Rohl et al (1976)	XRD, PLM, TEM, SEM	0.1-14% tremolite and anthophyllite (mostly fibrous) by weight in 10 of 20 consumer talc products tested
Paoletti et al (1984)	TEM	0.5-1.6% tremolite asbestos in 2 of 6 Italian cosmetic talc powders tested
		Trace to 0.15% chrysotile in 3 of 14, 18.7-21.7% anthophyllite asbestos and tremolite asbestos in 2 of 14, and 0.13% tremolite asbestos & chrysotile in 2 of 10 samples provided by the European Pharmacopeia
Blount (1991)	PLM	10 to 341 structures per mg amphibole fibers, needles, cleavages and 'prismatic pieces' in 9 of 14 samples of pharmaceutical and cosmetic-grade talc powders tested
Jehan (2004)	PLM	Qualitative identification of tremolite asbestos in 13 of 28, chrysotile in 12 of 28, anthophyllite asbestos in 3 of 28, and a mixture of asbestos fibers in 4 of 28 cosmetic talc powder products used in Pakistan
Floyd (2004)	TEM	0.20% anthophyllite asbestos by weight in Johnson's Baby Powder
Mattenklott (2009)	SEM	0.001-0.0073% asbestos by weight in 13 of 57 samples of talc powders sold on the German market from 1996 to 2005
Gordon et al. (2014)	PLM	1,840-1,104,000 fibers per gram asbestos in 50 of 50 historical samples of one brand of cosmetic talc powder tested (40 of 50 contained anthophyllite asbestos only, 4 contained tremolite asbestos only, 4 contained tremolite and anthophyllite asbestos, 2 contained tremolite, anthophyllite, and chrysotile asbestos)
	TEM	0.004-0.9% amphibole asbestos by weight in 9 of 9 samples of the same cosmetic talc product
Ilgren et al (2017)	TEM	3.687×10^6 tremolite asbestos fibers/gram in an authentic sample of commercial talc produced prior to 1975 from the talc mine in Val Chisone, Italy

Table 4: Summary of studies finding asbestos and/or talc in ovarian tissue from cosmetic talc use

Study	Tissue Weight Type	Test Method	Summary of Findings
Henderson et al. (1971)	n/a	TEM	Qualitative identification of talc in 10/13 ovarian tumors
			Qualitative identification of talc in 12/21 cervical tumors
Langer (1971)	n/a	Unknown	Qualitative identification of talc and chrysotile asbestos in Henderson et al (1971) samples
Heller, Westhoff et al. (1996)	Wet weight	PLM	26-464 talc particles per gram in 12/12 samples of benign ovarian neoplasms from 12 women with history of adult perineal talc use
			69-420 talc particles per gram in 11/11 samples of benign ovarian neoplasms from 12 women with history of talc diapering during infancy
			6-2,200 talc particles per gram in 6/7 samples of benign ovarian neoplasms from 12 women with no history of adult perineal talc use and an unknown history of other talc uses
		TEM	151,300-7,565,000 talc particles per gram in 5/12 samples of benign ovarian neoplasms from 12 women with history of adult perineal talc use
			151,300-1,600,288 talc particles per gram in 6/11 samples of benign ovarian neoplasms from 12 women with history of talc diapering during infancy
			63,042-1,669,000 talc particles per gram in 3/7 samples of benign ovarian neoplasms from 12 women with no history of adult perineal talc use and an unknown history of other talc uses
Cramer et al. (2007)	n/a	PLM & SEM	Qualitative identification of birefringent particles consistent with talc in pelvic lymph nodes of a 68-year-old woman with stage III ovarian papillary serous carcinoma and a 30-year history of perineal talc use

Exhibit B

ASPO American Society of Preventive Oncology

Menu

ASPO Abstracts

Genital Powder Use and Risk of Ovarian Cancer: A pooled analysis

Authors: Oâ€™Brien KM, Tworoger SS, Harris HR, Weinberg CR, Trabert B, Dâ€™Aloisio AA, Sandler DP*, Wentzensen N* on behalf of the Ovarian Cancer Cohort Consortium

Category: Lifestyles Behavior, Energy Balance & Chemoprevention
Conference Year: 2019

Abstract Body:
Purpose: The relationship between genital powder use and risk of ovarian cancer is not well-understood. Positive associations reported in case-control studies generally have not been confirmed in prospective cohort studies, which though not subject to recall bias, may lack sufficient power to identify modest associations. Methods: To address this, we pooled data from four large prospective cohort studies: Nurses' Health

Study, Nurses' Health Study II, Sister Study, and Women's Health Initiative Observational Study. Altogether, we had data from 250,641 women, including 2,073 who developed ovarian cancer. Results: Genital powder use was common (38% of non-cases ever used, versus 45% of cases) and varied somewhat by study sample (26%-53%). Using Cox proportional hazards models adjusting for potential confounders, we observed that ever powder use was associated with an 9% increase in the hazard of developing ovarian cancer, compared to never users (hazard ratio [HR] = 1.09, 95% confidence interval [CI] = 1.00, 1.20). The association was similar in frequent users (HR=1.10, 95% CI: 0.96, 1.25 for use >1/week versus none), but not among long-term users (HR=1.04, 95% CI: 0.84, 1.29 for >20 years of use versus none). The strongest association was observed among women with patent reproductive system, e.g. had a uterus and had not had tubal ligation, at the time powder exposure was assessed (HR=1.15, 95% CI: 1.03, 1.29). There were no clear differences by ovarian cancer subtype. Conclusions: This large, well-powered prospective study observed a weak association of genital powder with ovarian cancer risk, which appeared to be limited to women with patent reproductive tracts.

Keywords: ovarian cancer, powder, talc

Return to Abstract Listing

Exhibit C

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The effect of talc particles on phagocytes in co-culture with ovarian cancer cells

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ABSTRACT

Talc and titanium dioxide are naturally occurring water-insoluble mined products usually available in the form of particulate matter. This study was prompted by epidemiological observations suggesting that perineal use of talc powder is associated with increased risk of ovarian cancer, particularly in a milieu with higher estrogen. We aimed to test the effects of talc vs. control particles on the ability of prototypical macrophage cell lines to curb the growth of ovarian cancer cells in culture in the presence of estrogen.

We found that murine ovarian surface epithelial cells (MOSEC), a prototype of certain forms of ovarian cancer, were present in larger numbers after co-culture with macrophages treated to a combination of talc and estradiol than to either agent alone or vehicle. Control particles (titanium dioxide, concentrated urban air particulates or diesel exhaust particles) did not have this effect. Co-exposure of macrophages to talc and estradiol has led to increased production of reactive oxygen species and changes in expression of macrophage genes pertinent in cancer development and immunosurveillance. These ffndings suggest that in vitro exposure to talc, particularly in a high-estrogen environment, may compromise immunosurveillance functions of macrophages and prompt further studies to elucidate this mechanism.

1. Introduction

Macrophages (MΦ) phagocytize foreign particles and destroy malignant cells (Dunn et al., 2004); however, it is not often that these two activities are analyzed in the same context. This study was prompted by the epidemiological observation that cosmetic talc powder may be contributing to the risk of ovarian cancer (OC) (Penninkilampi and Eslick, 2018): we tested the hypothesis that interaction with talc particles compromises the MΦs by reducing their anti-tumor abilities.

Talc (hydrous magnesium silicate) is a mined substance considered 'inert' and used in cosmetic products including baby powder. Until 1970's talcum powder may have been contaminated with asbestos, which prompted the International Agency for Research on Cancer (IARC) to declare it carcinogenic to humans (class 1). Since approximately this time talc has been thought to be asbestos-free; nevertheless the IARC concluded that even talc not containing asbestos is possibly carcinogenic to humans (class 2b) (Baan et al., 2006), however the mechanisms were not entirely clear. Dozens of epidemiologic studies (Booth et al., 1989; Chang and Risch, 1997; Chen et al., 1992; Cook et al., 1997; Cramer et al., 1999; Godard et al., 1998; Harlow et al., 1992; Harlow and Weiss, 1989; Mills et al., 2004; Ness et al., 2000a; Purdie et al., 1995; Rosenblatt et al., 1998; Tzonou et al., 1993; Whittemore et al., 1988; Wong et al., 1999; Gertig et al., 2000; Hankinson et al., 1993) have identiffed a 35% increase in ovarian cancer (OC) risk for women who used cosmetic talc powder in the genital area (Cramer et al., 2016; Langseth et al., 2008). While the association is being actively debated (Muscat and Huncharek, 2008), a recent epidemiologic study suggests the association is stronger for women who were premenopausal or were postmenopausal but taking estrogen replacement therapy (Cramer et al., 2016). It is estimated that

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genital use of talc might account for 10–11% of OC cases in this country each year (Cramer et al., 1999); OC has a signiffcant contribution to the quality of life and surgical burden of disease.

The mechanism behind this link is unknown; but there are some insights. First, there is clear evidence that particles the size of talc, if they are present in the vagina, easily traverse to the upper female genital tract (Henderson et al., 1971; Heller et al., 1996; Edelstam et al., 1997; Sjosten et al., 2004; Cramer et al., 2007; McDonald et al., 2019a, 2019b). Second, the talc association was more apparent in pre-menopausal women and those postmenopausal women who were taking estrogen replacement therapy (Cramer et al., 2016) suggesting higher estrogen may ignite the pathogenesis (Berge et al., 2018). Third, some but not all experimental data suggest that talc particles are not completely innocuous: (NTP, 1993; Hamilton et al., 1984; Frazier-Jessen et al., 1996).

The epidemiologic data are at odds with the perception of talcum powder as a relatively inert, insoluble cosmetic substance that appears to have been well-tested and safe - as a chemical. However it is possible that talc, while it may not be directly mutagenic as a chemical compound (Boorman and Seely, 1995; Pickrell et al., 1989), is a hazardous factor as a particle. The approach and assays we used here stem from our prior interest in particulate matter and how macrophages interact with particles (e.g. (Zhang et al., 2015; Fedulov et al., 2008)); here we focused not on the process of carcinogenesis but rather on the immunotoxic effect of talc. Our hypothesis is that, in a high-estrogen environment, exposure to talc particles alters MΦ functions to permit increased survival of malignant cells. We postulate this could occur via a release of tissue-damaging factors (e.g. reactive oxygen species, ROX) and/or by compromising immunosurveillance abilities of the MΦs and their tumoricidal effectiveness.

2. Materials and methods

We used phagocytic murine cells lines J774 and IC21 and in some experiments RAW264.7 (ATCC; Manassas, VA) as phagocytes. These lines have been historically used to test the effects of female hormones on MΦs with success (Benten et al., 2001a; Pisetsky and Spencer, 2011; Hayashi et al., 1998). The J774 cells are 'chromosomally female' and thus are a better 'prototypical macrophage' for testing of estrogen effects. The IC-21 cell line was obtained by transformation of normal C57BL/6 mouse peritoneal macrophages (Mauel and Defendi, 1971). This line shares many properties with normal mouse MΦ and displays MΦ-speciffc antigens. IC-21 cells have phagocytic and cytolytic properties, can lyse tumor targets in-vitro (Crawford et al., 1990) and appear to be a terminally differentiated macrophage line (Walker and Demus, 1975; Walker and Gandour, 1980). Hence they are more relevant to OC, however they are 'genetically male' and thus may be less responsive to estrogen although they also express estrogen receptors and respond to estrogen stimulation (Benten et al., 2001b).

These cells were maintained in 100-mm Petri dishes in DMEM (for J774) or RPMI-1640 (for IC21) free of phenol red, supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL) and 10 mM HEPES.

Tumoricidal eff ciency of the MΦ was tested in a standard MΦ-tumor co-culture using the murine ovarian surface epithelial cell line (MOSEC) ID8 (Roby et al., 2000) provided by Dr. Katherine Roby (University of Kansas). ID8 cells most closely resemble human epithelial form of OC, which contributes to 90% of the cases (Roby et al., 2000; Greenaway et al., 2008). We have transduced these cells with an EF1α-GFP lentiviral construct (GenTarget, Inc.) containing Blasticidin-S deaminase and validated that ffuorescence was at acceptably stable level in preliminary studies (Fig. 5). These cells were maintained in DMEM with stable L-Glutamine (10-101-CV, Corning) and supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), Blasticidin S (10 μg/mL, Gibco) and 'ITS media supplement' containing 1.0 mg/mL recombinant human insulin, 0.55 mg/mL human transferrin (substantially iron-free), and 0.5 μg/mL sodium selenite (1:100) from Sigma-Aldrich.

Talc ($Mg_3Si_4O_{10}(OH)_2$, CAS Registry Number: 14807-96-6, USP grade, particle diameter < 10 μm, was obtained via JT Baker (Batch No: 0000184513) and is certiffed as asbestos-free. The particles were suspended in PBS and ffltered through 30 μm nylon mesh fflters (no visible loss of material has occurred). We did not use any commercial talc products.

Titanium dioxide (TiO_2), CAS Registry Number 13463-67-7, control particles (with mean particle size of ~1 μm) were a gift from Dr. L. Kobzik (Harvard School of Public Health, Boston, MA); these were used previously in our studies (Zhang et al., 2015; Fedulov et al., 2008).

Concentrated urban air particles (CAP) were obtained via Harvard School of Public Health particle concentrator (batch #816) and represent urban contaminants typically present in Boston air (Zhou and Kobzik, 2007; Imrich et al., 2000; Sigaud et al., 2007). They were suspended in PBS and used as is without ffltering or sterilization.

Diesel exhaust particles (DEP) were generously provided by Dr. Ian Gilmour at the U.S. Environmental Protection Agency and used by us in earlier studies (Fedulov et al., 2008; Gregory et al., 2017). They were also suspended in PBS and used as is without ffltering or sterilization.

All particles were of comparable "ffne" size although not identical, see Fig. 1. All particles were sonicated on ice to break up clumps using Qsonica Q55 probe sonicator.

Prior to experiments the cells were serum-starved for 24 h in Macrophage-SFM (serum free medium) (Gibco/Life Sciences). Adherent cells (in black-walled 96-well tissue culture treated plates, Corning) were then treated to 17-β estradiol (E2) (cell culture grade, Sigma Aldrich) in a range of concentrations from 10 to 0.0001 μg/mL; ethanol served as vehicle control. Talc (or control particle) suspension was added at the same time as estradiol in doses from 0.1 to 20 μg/well in dose-response experiments and in dose 10 μg/well otherwise. Detection of reactive oxygen species (ROX) was performed after 4 h via Cell ROX Green Flow Cytometry Assay (Molecular Probes). Viability analysis and cell count veriffcation were done after 24 h of incubation via staining with Annexin V and Sytox (Invitrogen). RNA isolation (via RNEasy kit, Qiagen) for gene expression testing was done after 24 h as well.

Co-cultures with MOSEC-GFP cells continued for 72 h; MOSEC-GFP cells were added at 5:1 (MΦ:MOSEC) ratio; particles were almost entirely phagocytized by MΦs by this time (Fig. 1 Panel 2) therefore we do not assume that MOSEC cells were exposed to particles. Medium with fresh estradiol (at the same concentration as the original) was replaced every 24 h to compensate for the estradiol decay. At 72 h the cells were detached (TrypLE, Lonza), washed once with phenol red – free RPMI containing 10% FBS and resuspended in ffow cytometry (FACS) buffer (PBS + 0.5% bovine serum albumin, Gibco) for analysis.

Flow cytometry was performed using MACSQuant Analyzer cytometer (Miltenyi) running MACSQuantify Software V2.11. Samples were gated based on their forward and side scatter to exclude the smallest debris and large clumps. The analysis region (gate) distinguished GFP-bright MOSEC cells from mildly autoffuorescent MΦ; we calculated percentage and mean ffuorescence intensity (MFI) in the GFP channel for the GFP-bright MOSEC region. The integral ffuorescence index was calculated as a product of 'percent positive' multiplied by 'MFI value' and reffects the ratio of surviving GFP-MOSEC cells normalized to the number of MΦs in combination with the extent of GFP transgene expression (Csepregi et al., 2018; Kamau et al., 2001). Talc particles did not contribute to the ffuorescence signal (Fig. 5).

Microscopy. To visualize engulfment of talc particles the cells were treated with talc suspensions as described. After 24 h the cells were detached by trypsinization, and centrifuged onto standard microscopy slides (VWR) via Cytospin II (Shandon). The slides were ffxed with methanol and stained by Diff-Quik, a version of Romanowski stain. The images were made on an Olympus BH-2 light microscope with attachments for polarized light microscopy and an Olympus Q-Color 5 camera. All pictures were taken with the same degree of partially

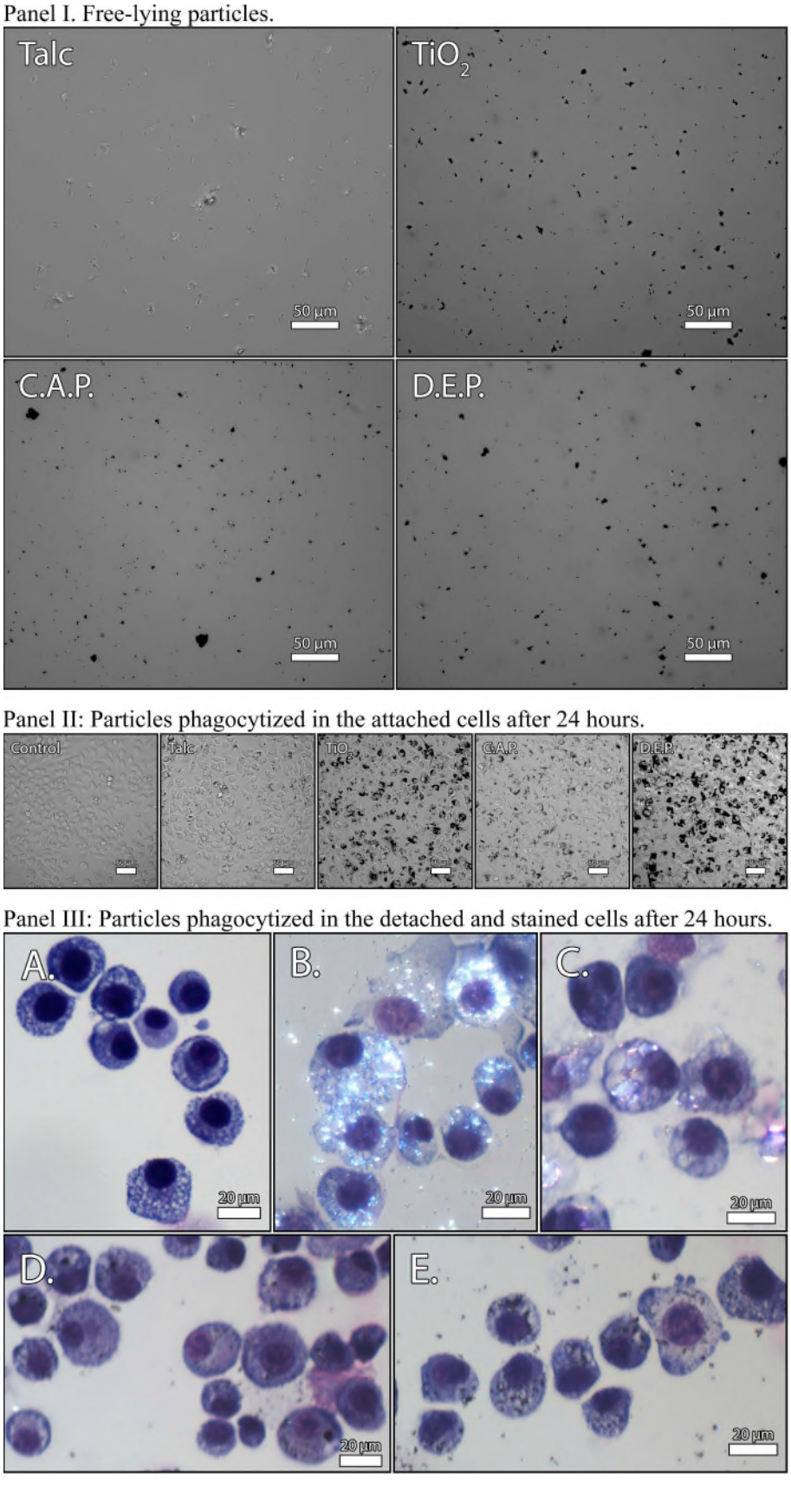

Fig. 1. Microscopic observation of particle phagocytosis.
Panel I: Free-lying particles. Particle suspensions were sonicated and plated freely in 300 µL of PBS, allowed to settle to the bottom for 1 hr and photographed using Nikon Eclipse Ti2 microscope. 400X.
Panel II: Particles phagocytized in the attached cells after 24 hours. Attached cells were treated to particle suspensions and allowed 24 hours to phagocytize. Nikon Eclipse Ti2; 400X.
Panel III: Particles phagocytized in the detached and stained cells after 24 hours. J774 cells without particles (A) and after 24 exposure to TiO_2 (B), talc (C), CAP (D) and DEP (E). Cytospin slides. Staining: Diff-Quik, original magniffcation for all images: 400X, on all images-bar: 20 µm. Olympus BH-2 light microscope with attachments for polarized light microscopy and an Olympus Q-Color 5 camera. All pictures were taken with the same degree of partially crossed polarizers so that black particles, birefringence of particles, and the cells could be seen. Note that talc and TiO_2 particles are birefringent, and single birefringent particles were seen in CAP and DEP preparations.

crossed polarizers so that black particles, birefringence of particles, and the cells could be observed.

To visualize the co-cultures with ffuorescent MOSEC cells with and without talc and control particles we used Nikon Eclipse Ti2 microscope with associated camera and software.

Gene expression proffling was achieved via Cancer PathwayFinder

RT2 Proffler PCR Array (Qiagen) which interrogates 84 cancer-pertinent genes using the CFX96 real-time PCR system (Bio-Rad) and CFX Manager 2.0 software (Bio-Rad). Raw Cq values for all genes (GOI) were normalized to an average of 5 housekeeping genes (HKG): Actb, B2m, Gapdh, Gusb and HSP90ab1 (NormΔCq = (Cq(GOI) - Ave Cq (HKG)). Expression values were obtained using formula 2^(-NormΔCq)*1000. These values were assembled into a matrix to become an input ffle for statistical analysis via TIGR Mev 4.9 (Saeed et al., 2003). Data were analyzed via Pavlidis Template Matching (PTM) method (Pavlidis and Noble, 2001) using the threshold p-value 0.05. In the heatmaps, red color indicates higher expression, green – low expression; row-normalized color intensity is proportional to the value for each gene in each sample.

All co-culture experiments were repeated more than 3 times. Each measurement was done in duplicate or triplicate. Triplicate RNA samples were selected from one representative experiment for gene expression analysis. Data are presented as Mean ± SEM. Data plotting and statistical analysis (other than array data) was performed using Excel 2007 (Microsoft) and Prism 7.02 (GraphPad Software); statistical signiffcance was accepted when $p < 0.05$. To estimate signiffcance of differences between groups we used the non-parametric Mann-Whitney *U* test, one-way or two-way ANOVA with Tukey, Fisher or HolmeSidak tests, or Kruskal-Wallis ANOVA with Dunn's or Dunnett's test as dictated by the number of groups, data normality and experimental question.

3. Results

3.1. Effect of talc and estradiol on the phagocytes

MΦs were treated with vehicle alone (ethanol), talc alone, estradiol (E2) alone, or the combination of E2 and talc (Fig. 1). Costimulation of MΦs with estradiol (E2) and talc produced an additive effect on ROX production (Fig. 2). While control TiO_2 particles were also phagocytized, the production of ROX was only slightly increased in J774 cells and not increased in IC21 cells (not tested in RAW264.7).

Gene expression proffling was performed via PCR-array aimed at detection of genes relevant in cancer pathways (see Qiagen PAMM-033ZD for the full list). Fig. 3B demonstrates a cluster of genes signiffcantly upregulated by talc in the two types of phagocytes: interestingly in J774 cells the effect of talc was prominent with or without E2, when in IC21 cells the co-effect of talc and E2 is better seen. When examining both cell types, we found patterns of similarity in the increased expression of this set of genes. Quite notably this cluster involves genes of extracellular, outer-membrane and releasable nature that are pertinent in carcinogenesis (see Discussion for details).

Fig. 3A demonstrates a cluster of genes co-inhibited by talc and E2, suggesting a strong co-effect of particles and the hormone, but also (more so for J774 cells) the effect of talc alone. Many of these genes encode intracellular factors pertinent to immunosurveillance, see Discussion for details. Many of the genes (but not all) were affected similarly in all three or in two out of the three cell types we tested.

In summary, talc alone and especially in combination with E2 produced changes in gene expression that may promote pro-tumorigenic environment and less eff cient surveillance (tumoricidal) activity of the macrophages.

Exposure of MΦs to talc or E2 did not lead to signiffcant increases in staining with Annexin V or Sytox (Fig. 4) or any noticeable changes in cell numbers in the 24 h period; the exceptionally high doses did occasionally decrease the viability of the MΦs (however slightly), hence we did not employ these concentrations in further experiments. Some variability in this staining is reported in Fig. S2.

3.2. Effect of phagocytes pre-treated with talc and estradiol on MOSEC ID8 cells

Wildtype MOSEC ID8 cells were transduced to express GFP under EF1a promoter. GFP^+ MOSEC ID8 cells were added for 72 h with addition of fresh E2 every 24 h of that period. Visualization of the co-culture was performed via an Eclipse Ti2 UV microscope (Nikon) with associated camera and software (Fig. 5). Detection of surviving GFP^+ MOSEC cells was performed via ffow cytometry.

MΦs were treated with particles in the presence of estradiol (E2) or vehicle, as before; in control samples talc was replaced by TiO_2, CAP or DEP particles.

In dose-response experiments we observed that talc and E2 have potentiated the effect, the magnitude varied depending on the MΦ cell line but the ffndings (Fig. 6) reffect that both substances had dose-response kinetics. IC21 cells did not appear as sensitive to E2 as J774.

Fig. 7 demonstrates that neither particle had a statistically signiffcant cytotoxic effect at 10 μg/well with or without E2 at 24 h (immediately before MOSEC cells were added). We report a microscopic observation that talc-treated cells appeared more fragile than any controls. Microscopically and via ffow cytometry we also report that most particles were phagocytized at this timepoint, with only single particulates remaining outside the cells. MOSEC-GFP cells were then added and co-cultured for 72 h.

Our key ffnding is presented in Fig. 8. A combination of talc and E2 (but not of control particles and E2) has allowed signiffcantly increased MOSEC-GFP readings compared to especially the vehicle-only control where most MOSEC cells were eliminated from the co-culture. Of note, talc alone tended to be effective (albeit not statistically signiffcant in all experiments or pooled data), especially for IC-21 cells. The particles alone (when no MΦs were present) did not signiffcantly affect the numbers of MOSEC cells after 72 h; there was a trend towards a slight decrease in cell numbers (Fig. 7G).

In a subset of experiments (with IC21 cells) we recorded the number

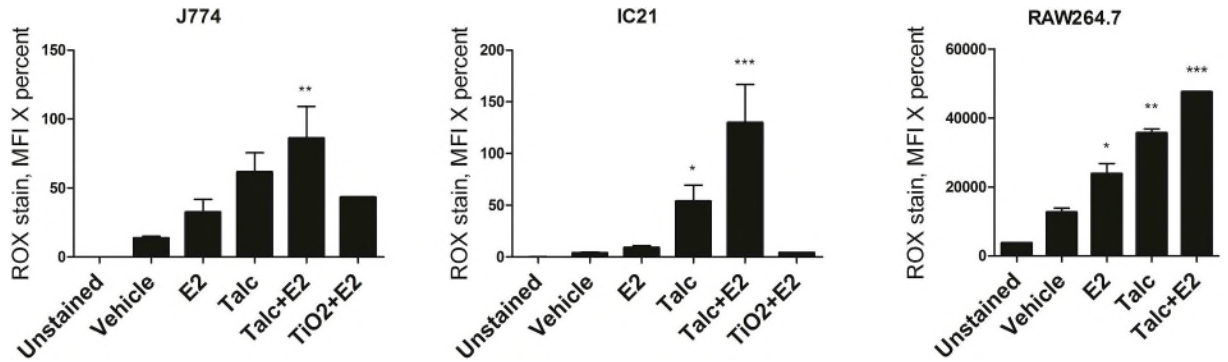

Fig. 2. Production of ROX at 4 h (ffow cytometry) was enhanced by either E2 or talc alone, the effect was additive. n = 2/group. *P < 0.05, **P < 0.01, ***P < 0.05 (Tukey).

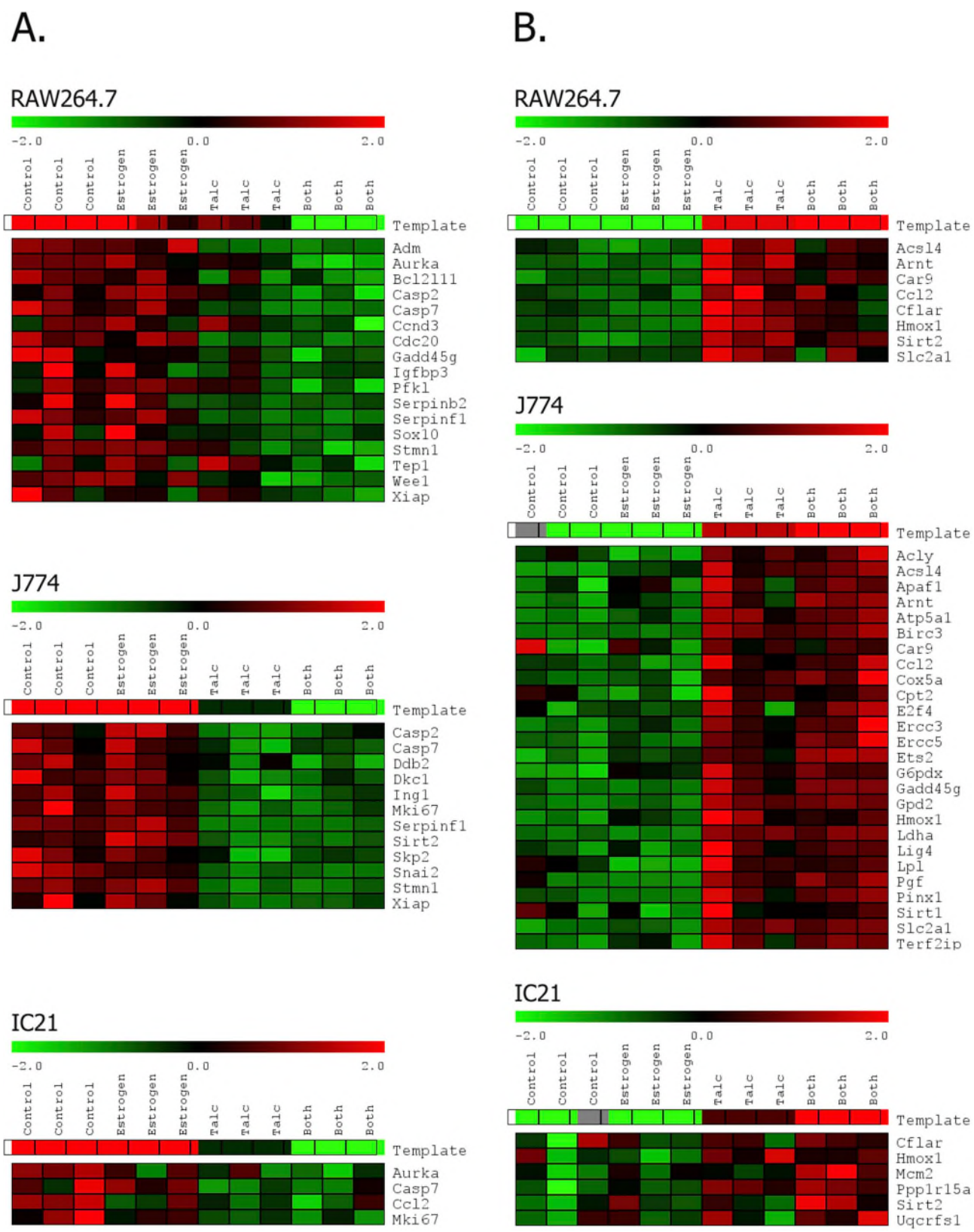

Fig. 3. PCR array profiling at 24 h exposure. Gene expression values were analyzed using Pavlidis Template Matching (PTM) with a threshold p-value of 0.05. Color is proportionate to gene expression (green = lowest, red = highest). A. Inhibitory effect: We aimed to identify genes most inhibited by the combination of estrogen and talc, but also affected by talc particles alone: the expression pattern of Aurka served as the template for RAW264.7 cell samples; matching template values (control: 0.9, estrogen: 0.9, talc: 0.4, both: 0) were used for J774 and IC21 cell samples. B. Stimulatory effect: Similarly, the template (control: 0, estrogen: 0, talc: 0.8, both: 1) aimed to identify targets most upregulated by the combination of estrogen and talc, but as well those increased by talc particles alone. Each sample tested is shown individually: N = 3 per group, total N = 36.

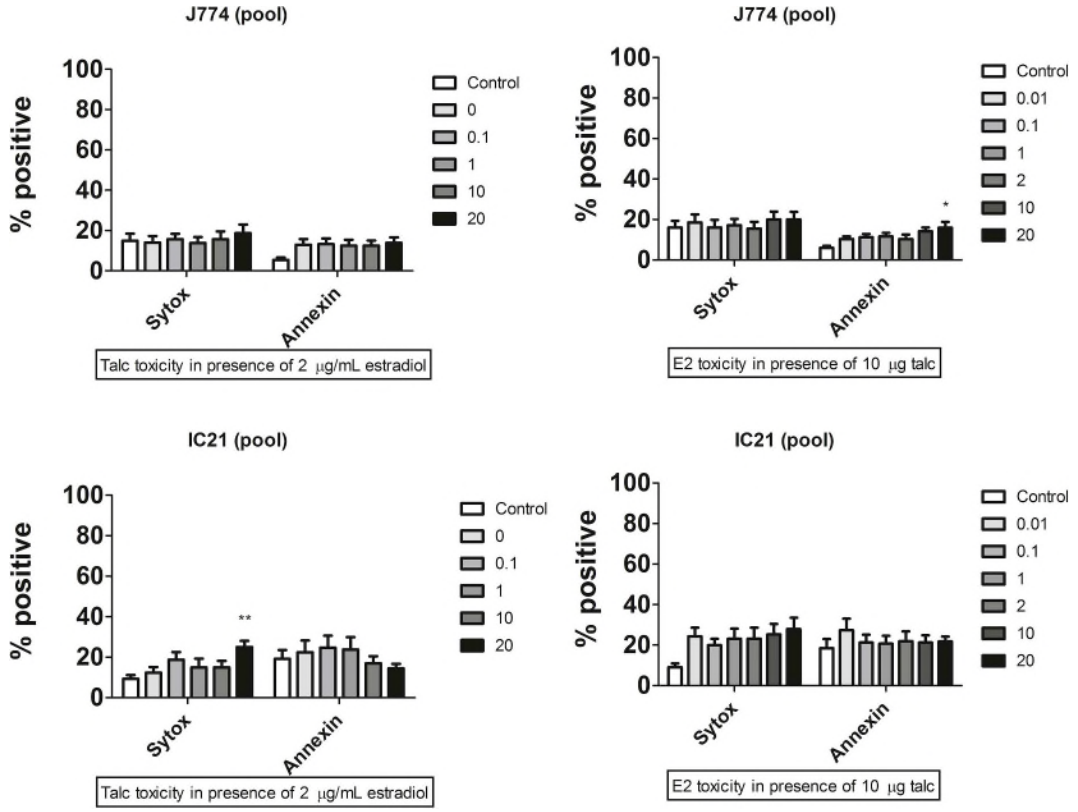

Fig. 4. Dose-response cytotoxicity analysis. Talc and E2 were not signiffcantly toxic to macrophages alone or in combinations used. J774 cells or IC21 cells were exposed for 24 h to either increasing doses of estradiol in presence of 10 μg/well of talc, or to increasing doses of talc in presence of 2 μg/mL of estradiol. Cells were stained for apoptosis and necrosis via Annexin V and Sytox assay kit; ffow cytometry determined the percentage of positive cells plotted here. Pooled data from three experiments are shown. n = 6 to 8 per group. *P < 0.05; **P < 0.01 (Dunn).

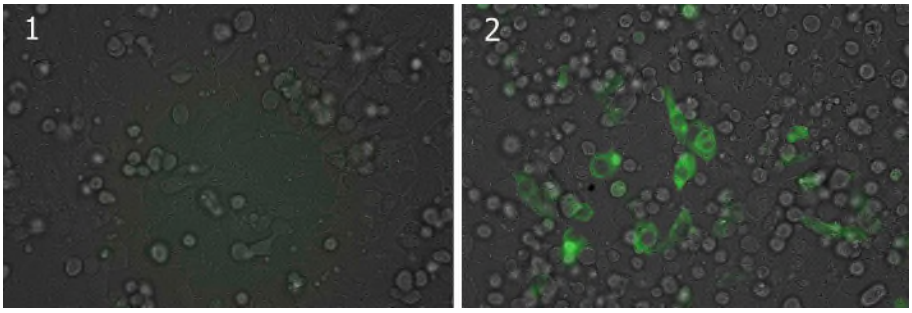

Fig. 5. Co-culture visualization: co-culture of IC21 macrophages with wildtype (1) or GFP$^+$ MOSEC cells (2) with talc photographed at the same setting in the FITC/GFP channel. Magniffcation X400; Nikon Eclipse Ti2.

of GFP$^+$ MOSEC (per microliter of cytometry buffer) and it was consistently (with ffuorescence) higher in the wells where MΦs were treated with Talc + E2 but not with TiO_2 + E2 or E2 alone (Supplementary Fig. 1).

In a validation experiment we used an alternative approach which did not involve a GFP transgene. RAW 264.7 MΦs were treated to talc particles and E2 similarly and co-cultured with wildtype MOSEC cells. After a 72-h co-culture the MOSECs were labeled with Calcein AM whereas the MΦs were labeled with anti- Ly6-C and anti-CD45; a proliferation ratio calculation revealed that the combination of talc and E2 allowed a larger proportion of MOSEC cells than either agent alone (Fig. S3).

In summary, a combination of talc and E2 especially, and in some cases talc alone, affected the MΦs to permit higher MOSEC-GFP survival.

C, D: Cytotoxicity analysis. IC21 or J774 cells were treated to particles alone or in combination with E2 for 24 h and analyzed via Sytox Green and Annexin V PE staining. N = 3 per group.

4. Discussion

This is the ffrst study linking the macrophage, talc particles and estrogen in a potential mechanism to explain the effect of talc behind the ovarian cancer statistics seen in epidemiology studies. Histology of surgically resected tissues shows that in the setting of known exposure, talc has been capable of migrating from the perineum to pelvic lymph nodes, ovary, fallopian tube, uterus and cervix (Cramer et al., 2007; McDonald et al., 2019a, 2019b); however carcinogenicity studies indicated that prolonged exposure to talc inhalation by some experimental animals does not induce cancer (Hamilton et al., 1984; Frazier-Jessen et al., 1996; Boorman and Seely, 1995; Pickrell et al., 1989) although some tumors, tumor-like morphological changes and

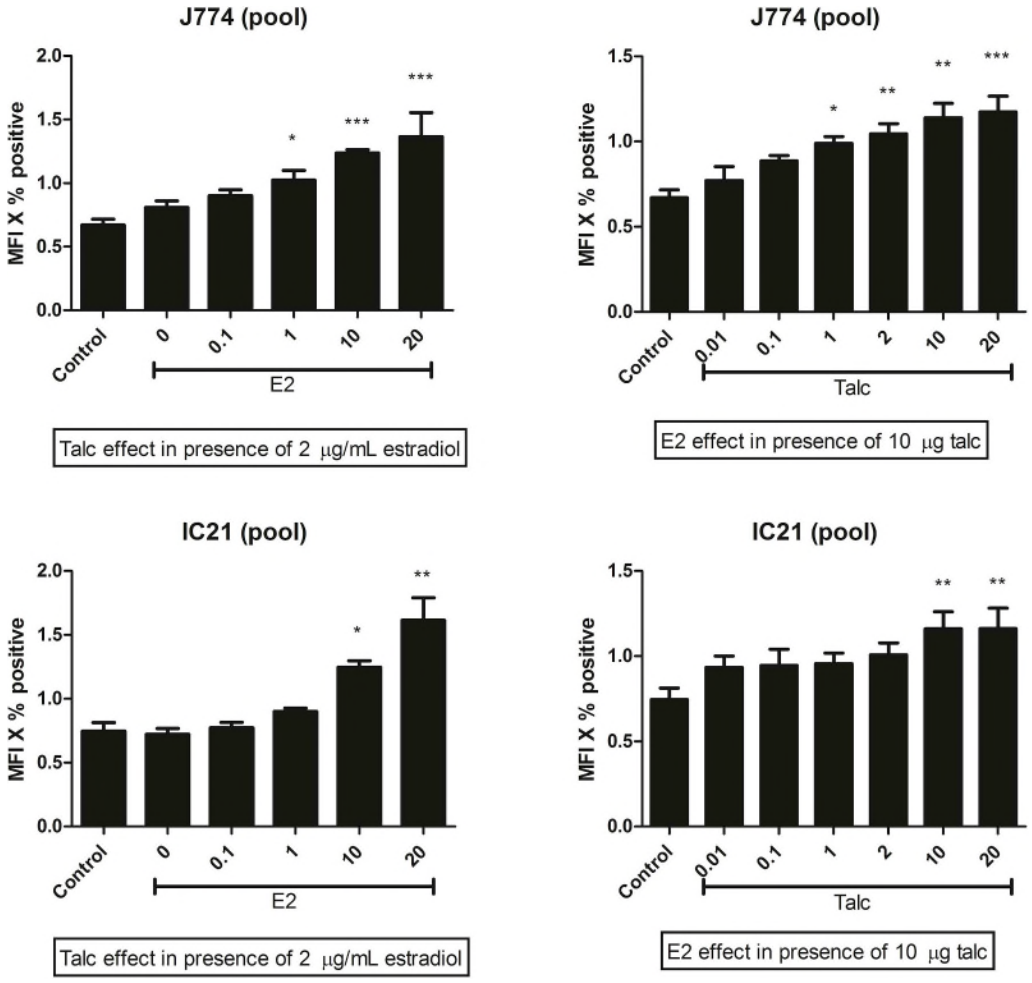

Fig. 6. Dose-response efiect in the co-culture. J774 cells or IC21 cells were exposed for 24 h to either increasing doses of estradiol in presence of 10 μg/well of talc, or to increasing doses of talc in presence of 2 μg/mL of estradiol. After preincubation, ffuorescent MOSEC ID8 GFP$^+$ cells were added for 72 h; medium and estradiol were replaced every 24 h. Flow cytometry at the end of co-culture recorded the percentage of GFP-positive cells and their MFI; the plots represent the product (MFI X percent). Data values from four experiments were normalized to average and pooled, n = 4/group. *P < 0.05; **P < 0.01; ***P < 0.005 (Dunn).

macrophage activation were reported (Shim et al., 2015; NTP, 1993; Hamilton et al., 1984).

Three particular lines of evidence argue that the estrogen milieu may determine the effects of talc: A) in humans, the talc association was more apparent in premenopausal women and those postmenopausal women who were taking estrogen replacement therapy (Cramer et al., 2016); B) in rodents, lung tumors developed in female, not male rats exposed to talc (NTP, 1993); and C) our own work indicating that estradiol (E2) affects MΦ uptake of particles (Zhang et al., 2015). Notably, there is no literature to suggest asbestos-free talcum powder causes any cancers in men.

Here we focused on the MΦ because A) MΦs are the ffrst to encounter and engulf talc particles; once phagocytized, these particles persist inside the MΦ (Goldner and Adams, 1977); B) MΦs are part of innate immunity responsible for the removal of aberrant, malignant cells (Dunn et al., 2004); they are especially active when primed (Hagemann et al., 2008). C) MΦs produce aggressive molecules capable of driving persistent tissue damage; and D) in patients with ovarian tumors, talc is observed within MΦs (Cramer et al., 2007).

Of note, the literature does not suggest an association of chronic pelvic inffammatory diseases with perineal talc use (Merritt et al., 2008), indicating that typical cytokine pathways are unlikley to make a signiffcant contribution (although inffammation can be a contributing factor in OC (Ness et al., 2000b)). Moreover, in a typical model, the MΦs are co-cultured over a large amount of tumor cells which leads to alternative activation (M2) phenotype, also called tumor-associated MΦs (Hagemann et al., 2006). These cells have distinct expression proffles and may be a suitable model to study processes in *established* tumors, whereas we are focused on the *onset* of the process. We emphasize that E2 pre-treatment does not affect this polarization *per se* (Wang et al., 2015); in our preliminary studies markers of M1 vs. M2 phenotype were unchanged (not shown). This is consistent with our hypothesis that combination of talc and E2 produces an effect in MΦs that is distinct from the heavily studied alterations.

Here we hypothesized that in a high-estrogen environment the talc particles alter MΦ function and decrease the killing of OC cells. We postulated this could occur via either a release of damaging factors that promote formation of aberrant (OC) cells, and/or via compromised immunologic surveillance (tumoricidal) ability of the MΦ, which could allow aberrant cells (that regularly appear in low numbers in the organism) to develop into clinical tumors. The latter premise was supported in part by a report that exposure of MΦs to talc can inhibit their phagocytic activity (Beck et al., 1987).

We found that talc and estradiol co-enhanced the production of ROX which participate in cell growth/proliferation, differentiation, protein synthesis, glucose metabolism and survival of malignant cells (Liou and Storz, 2010); ROX play important role in the pathogenesis of OC (Saed et al., 2017). This ffnding is consistent with in vivo data (Shim et al.,

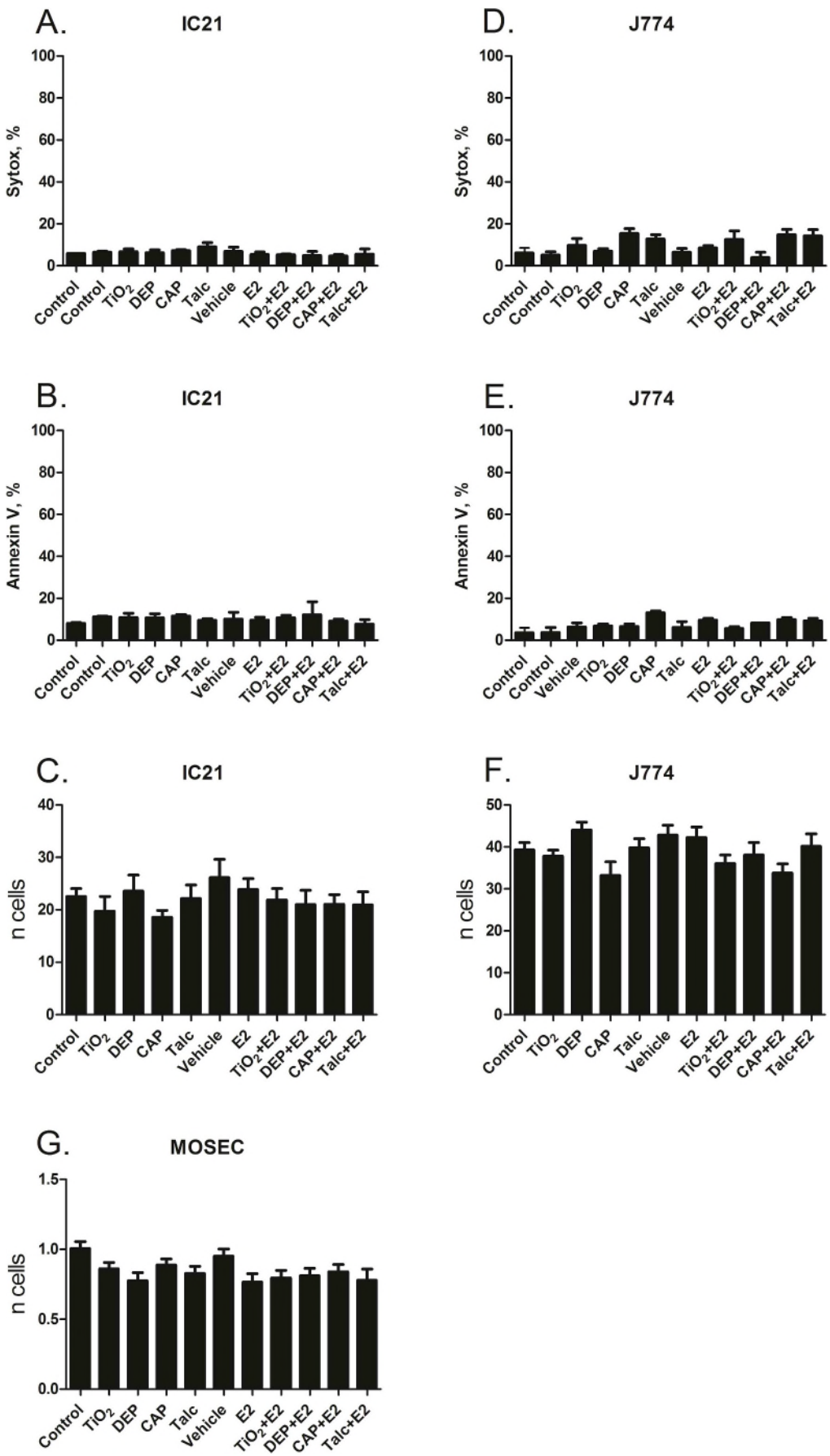

Fig. 7. Cytotoxicity analysis. IC21 cells (A, B) or J774 cells (D, E) were treated to particles alone or in combination with E2 for 24 hrs and analyzed via Sytox Green and Annexin V PE staining. n = 3 per group. Cell number: IC21 (C) or J774 cells (F) were visually counted in a haemocytometer after 24 hr incubation with particles and estrogen. Normalized average of 3 repeat experiments; N = 6 per group for IC21cells, n = 7 per group for J774 cells. G: The effect of particles on cell counts of MOSEC cells treated alone. MOSEC cells were visually counted in a haemocytometer after 72 hr incubation with particles and estrogen. Normalized average of 3 repeat experiments; n = 6 per group.

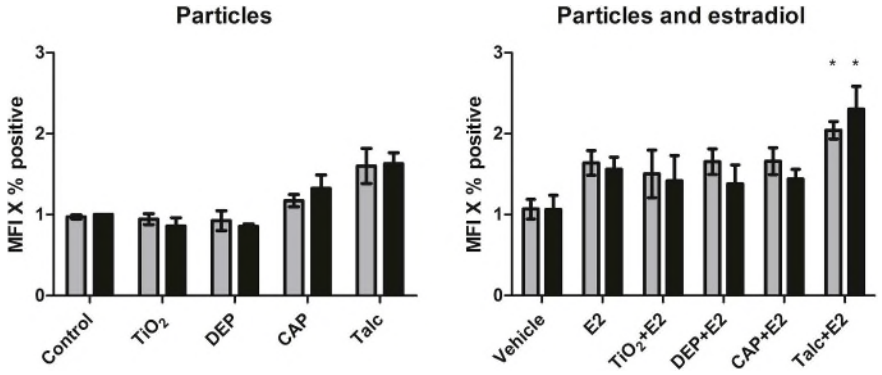

Fig. 8. The effect of talc and control particles in co-culture of MΦ and MOSEC cells. J774 cells or IC21 cells were exposed for 24 h to 2 μg/mL of E2 (or vehicle) and to 10 μg/well of talc, TiO_2, CAP or DEP particles, or combination. After 24-h pre-incubation, ffuorescent MOSEC ID8 GFP^+ cells were added for 72 h; medium and estradiol were replaced every 24 h. Flow cytometry at the end of co-culture recorded the percentage of GFP positive cells and their MFI; the plots represent the product (MFI X percent). Data values from three experiments were normalized to control and pooled, n = 3/group. *P < 0.01 vs. Vehicle (two-way ANOVA with Tukey or Holm-Sidak).

2015).

Moreover, talc alone, and to some extent in concert with estradiol has upregulated a cluster of genes that encode factors of releasable, extracellular or outer-membrane nature whose increase alters the extracellular milieu and contributes to tumor growth and metastasis: 1) Carbonic anhydrase *Car9*: enhances extracellular acidity and promotes tumor growth (Swietach et al., 2007, 2009); 2) HMOX1: macrophageal heme oxygenase-1 in tumor microenvironment can dictate cancer growth and metastasis (Nemeth et al., 2015); 3) Solute carrier family 2 facilitated glucose transporter member 1 (SLC2A1), a membraneous protein which promotes tumor cell proliferation and metastasis (Yan et al., 2015); 4) CFLAR, a gene that encodes Cellular FLICE-inhibitory protein (CFLIP), remarkably associated with carcinogenesis including OC (Lozneanu et al., 2015); 5) Sirtuin 2 (SIRT2) – a known therapeutic target in cancer (Jing and Lin, 2016).

At the same time, and perhaps more importantly, we found that talc and estrogen co-inhibited expression of a cluster of genes responsible for intracellular, internal proteins playing a role in anti-tumor immunosurveillance. The cluster includes 1) *Aurka* - Aurora kinase A, an intracellular protein which regulates proliferation and ability to develop the 'anti-tumor' M1 phenotype by the MΦs (Ding et al., 2015; Sica and Mantovani, 2012). 2) *Gadd45g* - Growth arrest and DNA damage-inducible 45, an intracellular protein involved in MΦ maturation; its deffciency causes less eff cient tumor immunosurveillance (Schmitz, 2013); although the expression change for this gene was cell-type dependent; 3) Casp7 (Caspase-7) – a protein playing role not only in apoptosis, but also important in MΦ phagocytosis: Casp7-deffcient macrophages show impeded completion of phagocytosis (Akhter et al., 2009); 4) CDC20 (Cell division cycle 20) - a regulatory protein shown to be upregulated in MΦ recruited into the tumor and, comparatively, downregulated in those MΦ not engaging with the tumor (Poczobutt et al., 2016); 5) Mki67 – a known proliferation marker; 6) Stmn1 (Stathmin 1) is involved in cell cycle regulation and its inhibition leads to a decrease in proliferation as it is involved in microtubule stability inside the cell (Rubin and Atweh, 2004); Stmn1 affects how MΦs are activated (Xu and Harrison, 2015); interestingly, micro-RNA targeting Stmn1 can be transferred from MΦs to tumor cells (Aucher et al., 2013); 7) XIAP (X-linked inhibitor of apoptosis protein) is important in resistance to cell death in MΦs and is generally involved in MΦ innate immune functions (Rijal et al., 2018).

In combination, our gene expression data indicate both an "outward effect": induction of releasable extracellular deleterious factors, as well as an "internal effect": inhibition of important intracellular factors. Hence, this exploratory proffling has provided us with a hypothesis that together these effects can create preferential conditions for the survival of OC cells in co-culture. Our expression proffling was not comprehensive: a whole transcriptome analysis is needed to uncover full details of the deregulation in the MΦs. We also did not aim to determine whether the changes we found are unique to talc. The focus of our experiments was to demonstrate whether talc is inert when phagocytized in high-estrogen milieu, and we conclude that it is not inert.

In co-culture experiments, we determined that co-exposure of the MΦ to talc and E2 permits higher numbers of OC (MOSEC ID8) cells to survive. We ffrst determined whether E2 and talc had any effect on the MΦ viability in monoculture. Talc or E2 had no toxic effect seen as either apoptosis or necrosis rate, aside of a slight change at 20 μg/mL E2; we have not used this excessive concentration further to assure that the viability of pre-exposed MΦs is the same in all samples. We noted that MΦs, which especially avidly phagocytized talc, had a slight morphological change in appearance, as seen in Fig. 1, which however did not lead to signiffcant changes in counts (Fig. 7).

Hence, we treated the MΦ with a combination of 10 μg talc per well and 2 μg/mL E2, and in subsequent co-culture the ffuorescence of GFP^+ MOSEC ID8 cells and their percentage were higher after 72 h (indicating their better survival) compared to controls where MΦ had been treated with vehicle alone or with either agent alone. When talc was replaced with control particles - TiO_2, CAP or DEP, the effect was also not seen (Fig. 8).

In dose-response experiments, the J774 cells, 'chromosomally female', appeared dose-responsively susceptible to the effect of E2 and talc, whereas the 'chromosomally male' IC21 were mostly susceptible to talc. In both cells, we note, even the lowest dose of E2 (1 ng/mL) has boosted (albeit not signiffcantly) the effect of talc.

Because the survival of MOSEC cells is dependent on the number of macrophages in a well we mostly relied on the ffuorescence parameters in the FITC/GFP channel, which takes into account the ratio of both cells types as well as the 'brightness' of the GFP transgene as a measure of viability (Csepregi et al., 2018; Kamau et al., 2001). However, in a subset of experiments we also physically counted the MOSEC-GFP^+ cells (see example from one experiment in Fig. S1) and used a transgene-independent method (Fig. S3) and these parameters gave consistent results.

We note that a bolus of 2 μg/mL of E2, although realistic, is likely at the higher end of concentration ranges. In normal mice and humans concentration of circulating E2 in serum is in the range of pg/mL to ng/mL (Wood et al., 2007; Zhang et al., 1999). However tissue levels of steroid hormones may exceed plasma by 20-30-fold (Batra, 1976; Akerlund et al., 1981; Straub, 2007) and ovarian tissue concentration of E2 is more than 100-fold higher than in serum (Lindgren et al., 2002). This may be an indication of why talc use is associated with ovarian cancer rather than at other sites. It is also worth noting that *in-vitro* bioavailability of the hormone from a single administration cannot be directly interpolated dose-wise to the sustained tissue exposure of the resident cells *in-vivo*. We also note that in modeling the effects of E2 sometimes even higher doses have been employed to make for a useful short-term model (Drew and Chavis, 2000).

Our report aims to establish the phenomenon of decreased anti-tumor (anti-MOSEC) activity of the phagocytes after talc and E2 combination pre-treatment; it also partly delineates what further studies are needed to elucidate the speciffc pathways involved into the inhibition of macrophageal activity. In our study we did not investigate carcinogenic properties of talc *per se*. Studies of other sources and batches of

talc as well as with other cell types are needed for a more comprehensive evaluation of the effect. Further research is needed to determine whether and to what extent the effect of talc on phagocytes exists in vivo, particularly in humans; these studies were beyond the scope of our project. We did not investigate whether the inhibited tumoricidal activity we discovered could entail an increased likelihood of tumor growth. However, we believe our ffndings can help reconcile the presumed innocuous nature of talc with epidemiological data on talc powder use and OC risk by suggesting that the effect can be mediated by the macrophages.

The ffndings of this study using phagocytic murine cell lines as prototypical macrophages and MOSECs as prototypical ovarian cancer cells suggest that in vitro exposure to talc particles, particularly in a high-estrogen environment, may compromise the macrophageal immunosurveillance functions. Control particles (titanium dioxide, concentrated urban air particulates or diesel exhaust particles) did not have this effect. Exposure of macrophages to talc and especially co-exposure to talc and estradiol has led to increased production of reactive oxygen species and changes in expression of macrophage genes pertinent in cancer development and immunosurveillance.

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Abbreviations

MOSEC	murine ovarian surface epithelial cells
DEP	diesel exhaust particles
CAP	concentrated urban air particles
TiO_2	Titanium dioxide
E2	17-β estradiol
ROX	reactive oxygen species
MΦ	macrophage

Data statement

Data supporting the ffndings may be obtained for academic purposes from the corresponding author upon a reasonable request through the editorial off ce after disclosure of the conffict of interest.

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Declaration of interest

The authors: AM, DJG, CCM, BWL, HW, LMR and AVF have no competing interests. JJG has served as an independent expert and provided expert testimony in talc and other environmentally related litigation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2019.108676.

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Exhibit D

Migration of Talc From the Perineum to Multiple Pelvic Organ Sites

Five Case Studies With Correlative Light and Scanning Electron Microscopy

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Key Words: Talc; Pelvic lymph node; Ovarian carcinoma; Scanning electron microscopy; Polarized light microscopy

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ABSTRACT

Objectives: *Genital talc use is associated with increased risk for ovarian carcinoma in epidemiologic studies. Finding talc in pelvic tissues in women with ovarian carcinoma who have used talc is important in documenting exposure and assessing talc's biologic potential, but tissue-based morphology studies have been rarely reported.*

Methods: *We report five patient cases with documented perineal talc use, each of whom had talc (by both polarized light and scanning electron microscopy) in multiple pelvic sites distant from the perineum. Six negative-exposure control patients were also analyzed.*

Results: *Talc particles were found in exposed patients, typically within two or more of the following locations: pelvic region lymph nodes, cervix, uterine corpus, fallopian tubes, and ovaries.*

Conclusions: *Our report adds new insights into the biologic potential of talc and suggests additional anatomic sites that should be closely examined for talc by oncologic surgical pathologists in the setting of perineal talc use.*

Of great current medical, public health, and medicolegal importance is the epidemiologic association of ovarian malignancy and the use of talc cosmetic products in the genital area. Relevant data from epidemiologic studies have shown a clear excess of women with ovarian malignancy who had used talc in their genital area prior to developing cancer compared with control women.[1-5] In 2006, the data were evaluated by the International Agency for Research on Cancer, which concluded that the data were sufficient to classify the use of talc (not containing asbestos) in the genital area as possibly carcinogenic (class 2B).[6] A recent summary of the epidemiologic data, as they existed cumulatively up to 2017, found that genital talc use may increase ovarian malignancy risk by about 30%.[7] A recent Health Canada assessment[8] resulted in a proposed recommendation that talc meets the criteria under paragraph 64(*c*) of the Canadian Environmental Protection Act and may constitute a danger in Canada to human life or health.

Although the hypothesis about talc and ovarian cancer took its origin, in part, from descriptions of talc in ovarian tissue,[9] the presence of talc in the tissues of the genital tract from women with ovarian malignancy has not been a component or focus of interest in epidemiologic studies. Published histopathologic data showing talc in pelvic organs are very limited. Finding talc in the tissues of exposed patients is part of a larger key principle: the

quantification of foreign material in tissue is critical to assessing the disease occurrence, causality, and severity related to that tissue (reviewed by Abraham[10]). This is perhaps best known for asbestos and mesothelioma or pulmonary fibrosis.[11] The most complete quantification is yielded through the digestion of a tissue sample, because this procedure uses much greater amounts of tissue than could be assessed in a histologic tissue section.[10] The procedure can be used to identify and quantify individual particles by transmission electron microscopy (TEM) or scanning electron microscopy (SEM), which are then characterized by energy-dispersive X-ray analysis (EDX), to verify that their elemental compositions are consistent with a specific type of foreign material exposure.[12] Applying TEM and/or SEM and EDX to tissue sections cut from paraffin blocks also yields meaningful quantification when the concentration of particles in tissue is high enough for this detection.[13,14] This procedure can also show the cellular location where the foreign material resides in a tissue section, for example, exogenous particles in macrophages within lymph nodes.[15] Foreign particulate exposure can be estimated by studying histologic tissue sections under polarized light microscopy, which shows birefringent material, including its size and shape.[16,17] Besides the utility of these methods from a scientific point of view, they have also been applied to medicolegal contexts stemming from injuries in various exposure settings, including asbestos.[10]

Tissue digestion must be paired with a good understanding of local histomorphology to be effective and for its data to be properly evaluated in context. Contamination from laboratory or other sources can potentially complicate tissue digestion procedures, in which the anatomic landmarks are necessarily dissolved in the process. A study by Heller et al[18] was done with tissue digestion and subsequent TEM on ovaries from 24 women having hysterectomy/oophorectomy to treat conditions other than ovarian malignancy. Birefringent particles were found in digestates of all 24 patients by light microscopy and talc in approximately half of the patients by TEM, and talc particle counts were unrelated to reported levels of perineal talc use. This suggested to the authors that unassessed exposures, including infant diapering, might help explain the apparently widespread nature of the finding. Also, even though the authors stated they used talc-free gloves, contamination from laboratory processing sources outside the authors' own environment could have also played a role, given the widespread occurrence of talc in many settings.

In a woman with ovarian carcinoma, looking for talc in benign residual ovarian tissue is a good initial way to find evidence of historical exposure, but in many cases, the ovary is largely replaced by the new growth of tumor, and in such situations, there is often little residual ovary found in resected specimens. A subset of authors from the present study has previously described a case report[15] in which a woman with serous carcinoma of the ovary and who had used talc in her genital area was shown to have talc in three of four examined pelvic lymph nodes. A subsequent recent study by the current authors[19] examined the presence of talc in a series of talc-exposed women with ovarian carcinoma and available pelvic region lymph nodes. This study showed that measurements of talc from digestion of nodes may be adversely influenced by contamination, which may spuriously raise measured talc counts and obscure differences between patients that are related to clinical history and that would otherwise be detectable and significant. Instead, our study demonstrated that polarized light microscopy and in situ SEM/EDX are recommended for the assessment of talc in lymph nodes and, by extension, other exposed tissues as well. The main reason is that in situ SEM/EDX preserves anatomic landmarks and so enables a much better assessment of what is likely to be contamination and what is not.

Until now, the presence of migrated talc in multiple locations in the female pelvis/genital tract in the same patient has not been reported. Such a finding, if present, would add new insights into the potential of talc present in the perineum to enter the upper genital tract and demonstrate the importance of a more careful examination of pelvic tissues from women with epithelial ovarian cancer to correlate with clinical history of talc exposure. We report here a series of five patient cases with documented talc exposure of the genital area and with surgically resected pelvic tissues that were examined by polarized light microscopy, SEM, and EDX for the presence of talc that had migrated from the perineum. These results are compared with examination of surgical material from six patients with ovarian carcinoma who had no genital exposure to talc.

Materials and Methods

Five patient cases were received for consultative purposes, each representing a patient with ovarian carcinoma and a history of perineal talc use. Clinical history, including surgical pathology reports, was provided for each patient with the consultative materials; also, additional history, including surgical history and perineal talc use, was obtained directly from the patients. All patient identifiers, including the 18 recognized Health Insurance Portability and Accountability Act identifiers,[20] were removed from the study data prior to final assembly of

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the data and publication. Histologic H&E-stained slides from the oncologic surgical treatment procedure (typically a total abdominal hysterectomy [TAH]/bilateral salpingo-oophorectomy [BSO] and various other auxiliary procedures) were provided by the outside hospital. All slides were analyzed with an Olympus BH-2 light microscope equipped with polarizing filter capabilities (analyzer and rotating polarizer with specimen slide in between). Each tissue slide was first reviewed to verify the histologic features, tumor type (if present), and tissue site (ovary, cervix, uterus, lymph node, etc). Then, each slide was scanned systematically and completely at ×200 under polarized light, and all birefringent particles were counted that were in the same plane of focus as the tissue. Birefringent particles were counted only if they were located more than a few cell widths' distance deep relative to the surface to avoid including any surface contamination in the analysis. Birefringent particles such as paper, organic debris, starch, and other clearly recognizable contaminants were not counted if they were in any way interpretable as related to the surface.

Paraffin blocks corresponding to histologic slides of interest were obtained from the treating hospital. The tissue blocks were handled with a procedure for in situ SEM/EDX, which was first described by Thakral and Abraham,[13] for assessment of particulate materials in paraffin-embedded tissue. The full details of this procedure as it was applied in our laboratory are available elsewhere.[21] Importantly, to protect against contamination, the tissue blocks were handled with particle-free gloves on precleaned surfaces and sectioned removing ~30 µm of tissue and paraffin using a rotary microtome with a fresh, clean stainless-steel blade. This sectioning was intended to remove any surface contamination from previous storage and handling. After the fresh surface was exposed, the block surfaces were washed in distilled, deionized water for 30 seconds to remove soluble surface materials such as sodium chloride and sodium phosphates used in processing for histology. The blocks were mounted for SEM examination and always kept in closed containers to limit any environmental contamination. A Hitachi SU6600 field emission SEM was used, with an Oxford EDX with Aztec version 2.0 to 3.3 software, EDX detector model X-Max 50 SDD, and electron beam penetration depth estimated at 2.5 µm, with an X-ray microanalysis range of 0.5 to 2.5 µm in depth. Talc particles were characterized by magnesium (Mg) and silicon (Si) peaks falling within 5% of the theoretical atomic ratio of 0.750 and atomic weight percent ratio of 0.649 (representative talc spectrum is shown in **Image 1**).

Because all patients in this study were born before 1970, which was the time point when talc manufacturers

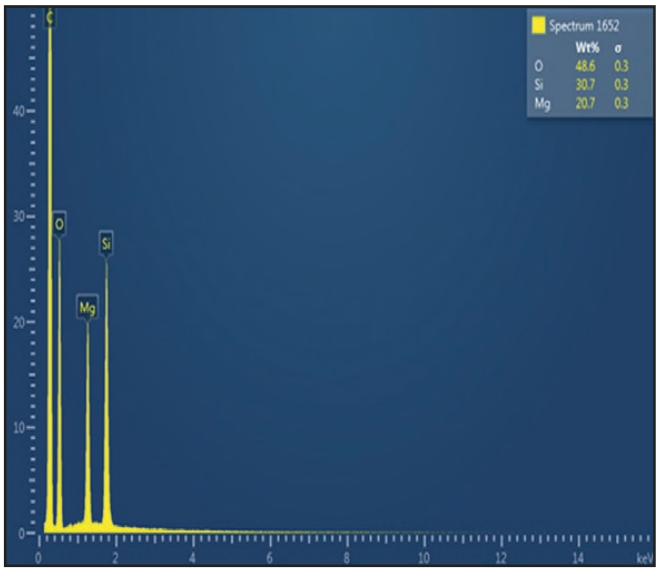

Image 1 Representative spectrum of talc, showing characteristic magnesium (Mg) and silicon (Si) peaks. The characteristic Mg-Si atomic ratio is 0.75 and atomic weight percent ratio is 0.649, and particles are considered to be talc if their Mg-Si ratio falls within 5% of this theoretical value (0.649).

claimed to voluntarily remove asbestos contamination from commercial talc preparations (establishing a cosmetic grade "free of asbestos" vs industrial grade that may contain it),[22,23] and because these patients had talc exposure extending across many years, we re-reviewed all SEM backscattered electron images generated on each patient specifically for fibers or fiber-like particles (defined as a 5:1 aspect ratio). We separately tallied and categorized them (one caveat being that plate-like particles, when viewed on edge, could give the impression of being a fiber, whereas with another orientation, they might not). The EDX spectrum for any particle meeting the fiber criterion was reexamined to determine its chemical composition, and where necessary, atomic weight percent calculations were done to determine fit (or lack of fit) with known classes of inorganic fibers.

To provide a set of nonexposed controls for the five patients in this case series, six patients with ovarian carcinoma were identified (see Results section and Supplementary Table 1; all supplemental materials can be found at *American Journal of Clinical Pathology* online) who were part of a large case-control study of ovarian cancer in eastern Massachusetts and New Hampshire.[3] Patients completed structure interviews and provided written informed consent allowing for review of pathologic material from their surgery. The study was approved by the Dana Farber/Harvard Cancer Center Ethical Review Panel. Patients were selected who stated that they had not used talc, either in their perineal area or as a general body

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powder. These patients had a distribution of tumor types (five serous carcinomas, one endometrioid carcinoma), ages (47-58 years), and remote surgical history (ie, prior to the development of cancer) similar to the five patients in the main study, and all had undergone TAH/BSO as part of their surgical oncologic treatment. In addition, patients from the Brigham and Women's Hospital were selected to facilitate retrieval of archival materials. H&E slides were examined by regular and polarized light microscopy. A count of birefringent particles was made by systematic, complete review at ×200 of each H&E slide under polarized light microscopy, the same as for the patient slides in the main study. Subsequently, and also similar to the main study, tissue blocks were examined with SEM/EDX, using the same in situ method previously described, and with all talc or other backscattered electron imaging-positive particles characterized.

Results

Table 1 shows key clinical details of the five talc-exposed women in this series. Ages fell within a fairly narrow range (47-59 years). Three patients had serous carcinoma, one endometrioid carcinoma, and one clear cell carcinoma. All these histologic types have been identified as being included in the general increase in risk with talc exposure in epidemiologic studies.[5] Pathologic International Federation of Gynecology and Obstetrics staging ranged from IA (one patient) to IC (one patient) to IIIC (three patients). All patients had BSO, and four of the five had accompanying TAH. Four of five had pelvic region lymph nodes excised for staging and/or treatment purposes.

Table 2 shows the findings from polarizing light microscopy of key sections from the talc-exposed patients' resected tissues. All patients had significant numbers of birefringent particles in tissue sections from two or more pelvic region sites, ranging from two (case 1, exocervical soft tissue and right pelvic lymph node) to four (case 2, with large numbers of particle accumulations in uterine serosa, pelvic lymph nodes, ovaries [right > left], and the fibromuscular tissue surrounding the right fallopian tube). Case 3 showed birefringent particles in the uterine serosa, bilateral fallopian tubes, and ovaries. Cases 4 and 5 had birefringent particles in the tissues of multiple

Table 1
Talc-Exposed Patients' Clinical Histories

Case No.	Age, y	Tumor Type	Pathologic Stage	Surgical Procedure Type	Exposure History (Talc Years)[a]	Comments
1	47	Endometrioid carcinoma, G3 (poorly differentiated)	pT1c N0 MX (FIGO stage IC)	TAH/BSO with multiple pelvic/para-aortic lymph node excisions, with omentectomy/ appendectomy (February 2009)	42	No history of surgeries prior to TAH/BSO
2	50	Serous carcinoma, high grade	pT3c N1 MX (FIGO stage IIIC)	TAH/BSO with multiple pelvic/para-aortic lymph node excisions, with omentectomy (October 2013)	31	Diagnostic cervical, pelvic mass, and pelvic lymph node biopsies performed a few months prior to TAH/BSO; tubal ligation at age 20 years
3	59	Serous carcinoma, high grade	pT3c NX MX (FIGO stage IIIC)	TAH/BSO with omentectomy (June 2010)	58	Diagnostic omental mass biopsy 2 weeks prior to TAH/ BSO; tubal ligation and cesarean section at age 32 years and cholecystectomy at age 45 years
4	49	Serous carcinoma, low grade	pT3c N0 MX (FIGO stage IIIC)	BSO with multiple pelvic/para-aortic lymph node excisions, with omentectomy, appendectomy, and right hemi-diaphragmectomy (March 2013)	31	No history of surgery prior to BSO
5	56	Clear cell carcinoma, grade 2	pT1a N0 MX (FIGO stage IA)	TAH/BSO with multiple pelvic/para-aortic lymph node excisions, with omentectomy/ appendectomy (March 2009)	51	No history of surgery prior to TAH/BSO

BSO, bilateral salpingo-oophorectomy; FIGO, International Federation of Gynecology and Obstetrics; TAH, total abdominal hysterectomy
[a]Talc year = daily (at least) application of talc-containing hygiene product to the genital area for 1 year. Patients 3 and 5 had reportedly experienced talc exposure since birth and/or early in infancy.

pelvic sites (fallopian tubes, ovaries, pelvic region lymph nodes), but due to the logistics of case review, processing, and send-out, we were not able to quantify these retroactively with additional light microscopy after the in situ SEM data had been obtained on the same blocks.

Table 3 shows in situ SEM/EDX data on the same patients as in Table 2, with 28 total blocks (across the five patients) examined by SEM/EDX and included in our case series (most, but not all, of the blocks in which birefringent particles were seen subsequently proceeded to electron microscopy). As is shown, there were generally substantial talc particle counts in the same tissue blocks corresponding to where birefringent particles were identified by light microscopy. For example, markedly high light microscopic particle counts in Table 2 for case 1 (cervix and right pelvic lymph node), case 2 (right fallopian tube), and case 3 (uterine serosa) were all matched by high talc particle counts by SEM/EDX for the corresponding cases and tissues in Table 3. Comparative examination of the data in Tables 2 and 3, particularly the pairs

Table 2
Polarizing Light Microscopy Findings in Pelvic Tissues From Five Talc-Exposed Patients

	Distribution of Birefringent Particulates Within Tissue (Particles/Histologic Section), No.			
Case No.	Lower Tract (Uterus/Cervix)	Fallopian Tube	Ovary	Lymph Nodes
1	Cervix: >100	Left tube: 3 Right tube: 3	Left ovary: 6	Right pelvic node: >500 Left pelvic node: >50
2	Anterior cervix: 6 Posterior uterus: >50	Right fallopian tube: >50, mainly in fibromuscular tissues near the tube	Right ovary, first block: 13 Right ovary, second block: 35 Right ovary, third block: 3 Left ovary, first block: 1 Left ovary, second block: 6	Right pelvic node: >100 Left pelvic node, first block: >200 Left pelvic node, second block: >100 Left pelvic node, third block: >100 (Note: first through third blocks are together one node.)
3	Uterus: >200	Right tube: 15 Left tube: 14	Right ovary: 27 Left ovary: 11	None surgically resected
4	Tissue type not made available	Birefringent particles seen in left fallopian tube (one block) and right fallopian tube (two blocks); exact counts not available	Birefringent particles seen in left ovary (two blocks) and right ovary (two blocks); exact counts not available	Birefringent particles seen in right pelvic lymph nodes (four blocks) and left pelvic lymph node (one block); exact counts not available
5	No birefringent particles seen in tissues	Birefringent particles seen in left fallopian tube (one block) and right fallopian tube (one block); exact counts not available	Birefringent particles seen in right ovary (one block) and left ovary (one block); exact counts not available	Birefringent particles seen in left pelvic lymph nodes, two blocks (three nodes total by gross examination report); exact counts not available

Table 3
In Situ Scanning Electron Microscopy Findings for Pelvic Tissues in Five Talc-Exposed Patients

	No. of Talc Particles Found in Each Tissue Block by In Situ Scanning Electron Microscopy/EDX			
Case No.	Lower Tract (Uterus/Cervix)	Fallopian Tube	Ovary	Lymph Nodes
1	Cervix: 52	Left tube: no SEM done Right tube: no SEM done	Left ovary: 8	Right pelvic node: 65 Left pelvic node: 61
2	Anterior cervix: 1 Posterior uterus: 53	Right tube: 31	Right ovary, first block: 2 Right ovary, second block: 51 Right ovary, third block: 0 Left ovary, first block: 1 Left ovary, second block: 3	Right pelvic node: 18 Left pelvic node, first block: 43 Left pelvic node, second block: 35 Left pelvic node, third block: 24 (Note: first through third blocks are together one node.)
3	Uterus: 36	Right tube: 2 Left tube: 1	Right ovary: 24 Left ovary: 0	None resected
4	Not examined by SEM	Not examined by SEM	Right ovary, first block: 4 Right ovary, second block: 0	Right pelvic node: 28 (one block examined)
5	Not examined by SEM	Right tube: 0	Right ovary: 8 Left ovary: 0	Left pelvic node: 13 (one block examined)

EDX, energy-dispersive X-ray analysis; SEM, scanning electron microscopy.

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of tissue blocks (generally in cases 1 through 3) for which both polarized light microscopy and SEM/EDX numerical particle counts were available, showed an *r* value of 0.675 and a *P* value of .002 by linear regression analysis. Where direct comparisons between the counts in Tables 2 and 3 were able to be made (generally the first three cases), counts of birefringent particles by light microscopy were generally higher than the corresponding counts by SEM. This can be explained by the finding of other nontalc foreign material in the blocks by SEM/EDX. EDX analyses were performed of backscatter-positive particles seen in these blocks. Across the 28 blocks, this yielded an aggregate total of 503 talc particles and 945 foreign nontalc particles. Of the latter, most (802, 85%) were nonspecific mineral particles consisting generally of Si in various combinations with sodium (Na), Mg, and especially aluminum (Al). Where Mg and Si predominated in this group, the spectral peak ratio fell outside the atomic weight percent range (0.649% ± 5%) expected for talc, so they were not classified as such. Occasionally, silicon oxide particles were identified by SEM/EDX, which exhibits birefringence.[17] The remaining exogenous particles consisted of various metals, either alone or in various combinations, most notably copper, chromium, Al, titanium, zinc, nickel, and manganese. Iron (Fe) was often combined with some of these metallic particles. Besides talc and exogenous metals and minerals, the other broad category of particles seen in the case analyses included endogenous particles, often in the form of dystrophic calcification, which is common in serous ovarian malignancy. Particles with calcium (Ca), Na, phosphorus (P), carbon, potassium (K), and Fe in various combinations were considered endogenous. No asbestos fibers or ferruginous bodies were found in the analyses. Based on the data, the nonspecific mineral particles accounted for many of the birefringent particulates seen under light microscopy that were not talc. Such particles can be encountered in everyday living and may presumably gain access to the perineum and associated lymphatics in similar ways to talc. Based on data from Jurinski and Rimstidt[24] for talc vs silica, these nonspecific silicates could be reasonably expected to have a slow dissolution rate (years or decades) and a long retention time in tissue.

Tissue macrophages were a key particle location for many sites and thus a key part of the tissue response to the migrated talc. Such cells, with cytoplasm filled with birefringent particles, were seen in the cervix (case 1) and the uterine serosa and pelvic region lymph nodes of multiple cases. In rare instances, the affected macrophages were seen to coalesce into multinucleate giant cells as part of the inflammatory response. Affected macrophages often had grayish, faintly ground-glass cytoplasm and were sometimes accompanied by a mix of other chronic inflammatory cells (eg, in the soft tissues near the fallopian tube in case 5). Birefringent material was often seen localized near small vasculature, particularly in the uterine serosa (cases 1 and 3) and soft tissue near fallopian tubes (cases 2 and 5). For extranodal talc migration sites, the concomitant presence of lymphatic vessels was strongly suspected, but this was often difficult to ascertain histologically since these vessels may be nonpatent and/or otherwise hard to see in tissue sections. Of note, for three of the four patients who had pelvic region lymph nodes resected, none of their talc-positive lymph nodes had concomitant metastatic malignancy. However, case 2 had two pelvic region lymph nodes (represented on slides as multiple lymph node profiles) with both metastatic tumor and abundant birefringent particles in macrophages, often existing close to each other. An example is shown in **Image 2**. Because both are regarded as migrating via lymphatic pathways, their coexistence in one of our patient cases was not a surprise. **Image 3** emphasizes this point from a different point of view by showing regular H&E (Image 3A) and polarized light microscopy (Image 3B) of the same view of uterine serosa in case 3. Several lymphovascular spaces are present, a larger one of which is highlighted with the arrows and is seen to contain birefringent material in Image 3B. EDX of this patient's uterine serosa tissue showed that this birefringent material was talc (see next paragraph).

Image 4, **Image 5**, **Image 6**, **Image 7** and **Image 8** (pertaining respectively to cases 1 through 5 in Tables 1-3) show representative correlative polarized light and SEM (with backscattered electron imaging) micrographs. For each case, EDX analysis of most of the backscattered image-positive particles (typically 1-10 µm diameter) showed the characteristic spectrum of talc in Image 1, thus confirming that most of the birefringent material seen by polarizing light microscopy in these particular areas was, in fact, talc. Considering each figure individually, Image 4 shows birefringent material clustered in macrophages in deep exocervical fibrous tissue and comparable particle morphology in the same region on backscattered electron SEM imaging. Similar correlative morphology is seen in the same figure for macrophages within pelvic lymph node tissue. The exocervical tissues and lymph node show rather unremarkable macrophage morphology when reviewed by light microscopy without polarization. Image 5 shows birefringent particle accumulations in the uterine serosa (both macrophages and soft tissue and near vascular spaces) and fallopian tube peripheral tissue, ovary, and lymph node tissue (the latter frequently in macrophages), with corresponding morphology in the SEM backscattered electron images. Image 6 shows

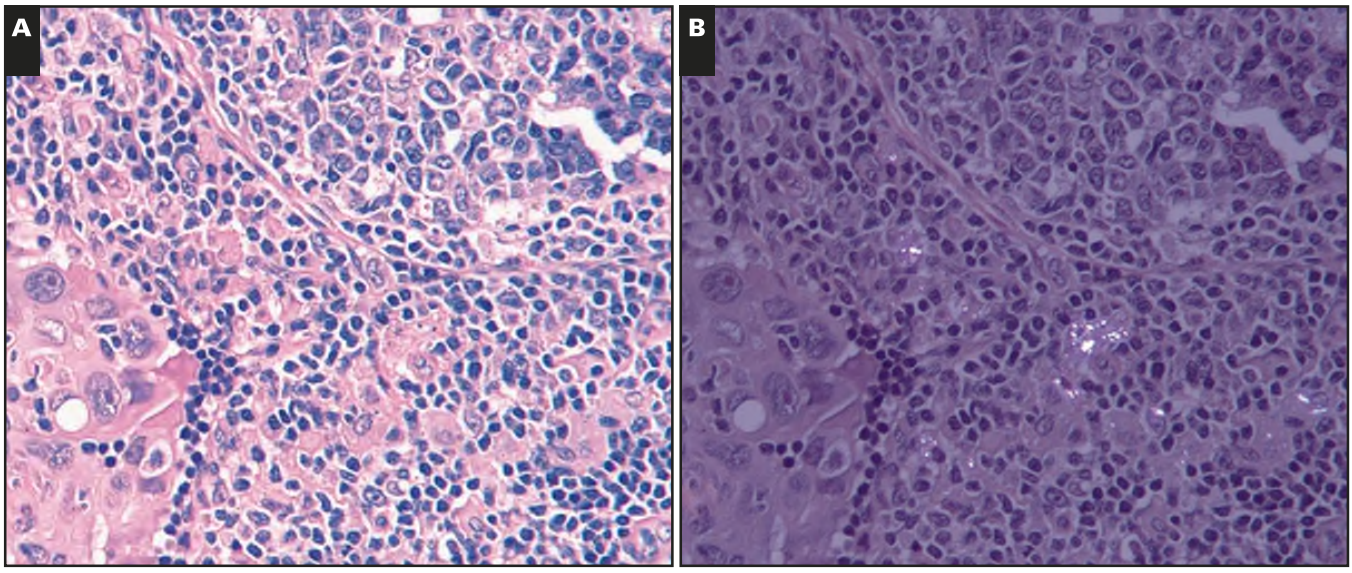

Image 2 Regular light microscopy (**A**) and polarized light microscopy (**B**) of left pelvic lymph node in patient 2, same fleld of view, showing juxtaposition of birefringent particles in macrophages, metastatic carcinoma, and uninvolved lymph node parenchyma. This particular area was not analyzed by scanning electron microscopy, but based on the flndings in other histologic regions, much of this birefringent material is likely talc. (H&E, ×400)

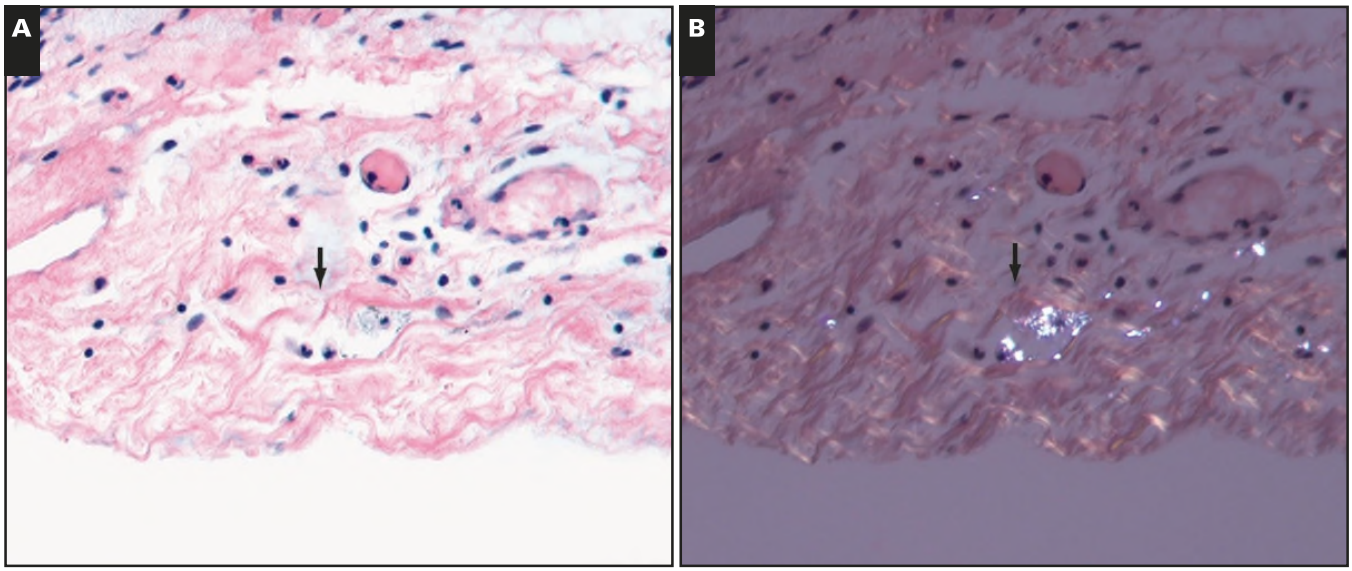

Image 3 Regular light microscopy (**A**) and polarized light microscopy (**B**) of the uterine serosa in patient 3, same fleld of view, showing serosal flbrovascular tissue and abundant birefringent particles that are seen in one lymphvascular space (arrows). This serosal birefringent material was shown to be talc by scanning electron microscopy/energy-dispersive X-ray analysis (see Image 6). (H&E, ×200)

uterine serosa with numerous birefringent particles within soft tissue and macrophages, as well as ovarian stroma showing a birefringent particle within soft tissue but close to a blood vessel, with corresponding SEM images showing backscattered electron-positive particles. Images 7 and 8 show birefringent particles in pelvic region lymph nodes with corresponding backscattered electron-positive positive SEM images, as well as birefringent particle(s) in auxiliary sites: ovary (Image 7) and soft tissue from around the fallopian tube (Image 8). The latter was notable for a mixed chronic inflammatory infiltrate in and around the exogenous material.

Review of the backscattered electron SEM images from all 28 tissue blocks from all five patients (typically there were around 50-250 SEM images generated on each block) showed a total of 52 fibers or fiber-like particles,

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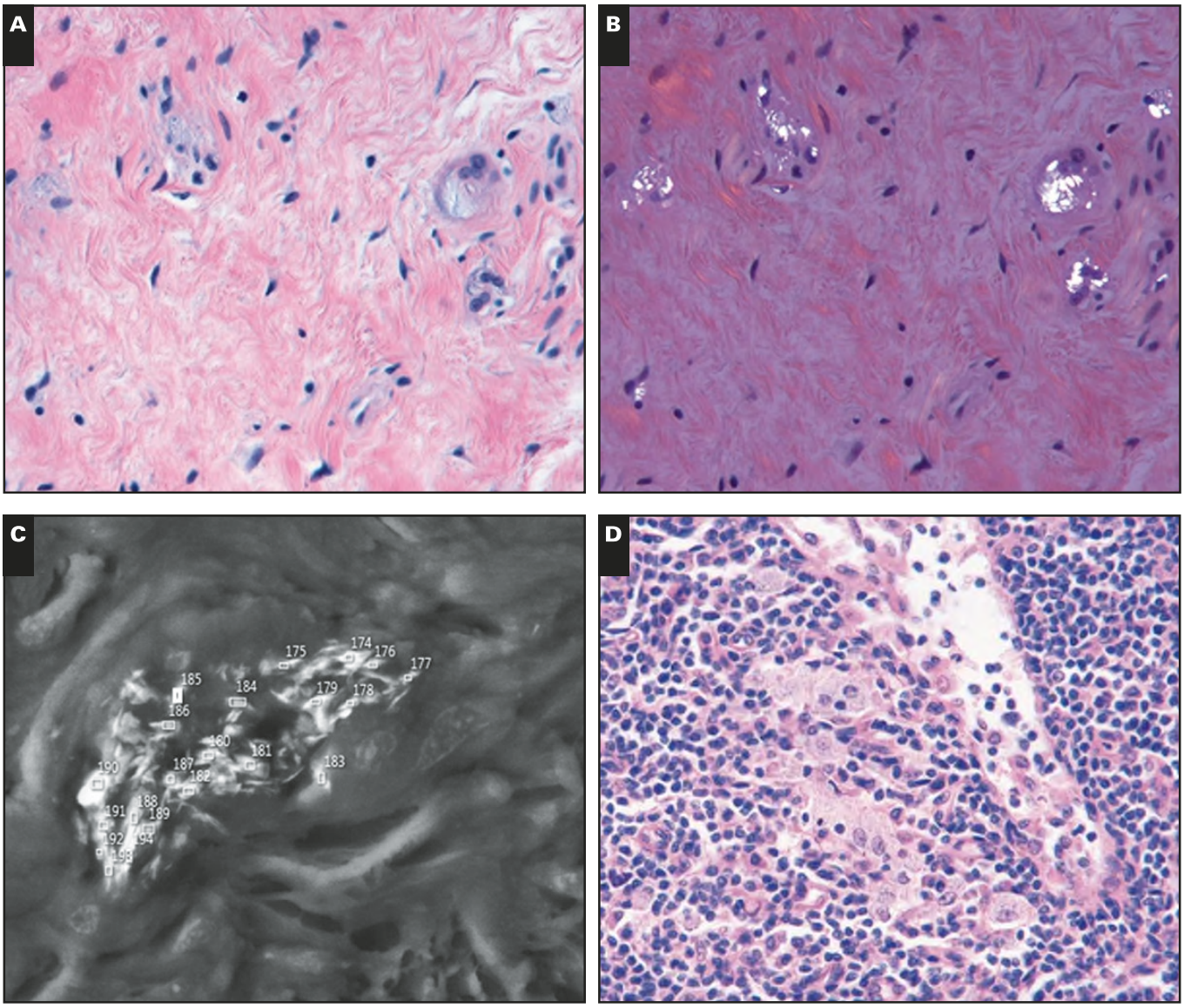

Image 4 Representative photomicrographs for patient 1. **A**, Deep exocervical soft tissue with collections of macrophages in dense collagenous tissue. A few macrophages were multinucleate and showed slightly glassy grayish cytoplasm (H&E, ×400). **B**, Same histologic fleld as **A**, under polarized light microscopy, showing collections of macrophages with numerous birefringent cytoplasmic particles 1 to 10 μm in diameter (H&E, ×400). **C**, Scanning electron microscopy (SEM, ×500) with backscattered electron imaging from the same general area as in **A** and **B** but a different histologic section, showing numerous backscattered electron-positive particulates within the cytoplasm of macrophages, similar to **A**, the majority of which had a spectrum characteristic of talc. **D**, Right pelvic lymph node with aggregates of intranodal macrophages (H&E, ×400).

of which 18 (35%) were talc, 18 (35%) were nontalc mineral silicates (typically Al-Si often in combination with other cations), six (11%) were metals or combinations of metals, and 10 (19%) were endogenous (various combinations of Na, P, sulfur, Ca, K, and Fe). Most of the identified fiber-like particles had aspect ratios approximately or slightly greater than 5:1 (the threshold we used), but there were four fibers identified with long aspect ratios (>10:1) and strongly parallel sides. Three of these were found in the right ovary of patient case 2, and the fourth was found in the left fallopian tube of patient case 3. By EDX, these fibers were aluminum silicates with Mg and Ca and, in two of the fibers, also Fe. Atomic weight percent calculations on these fibers showed that the Mg/Si and Ca/Si ratios were far outside the ranges expected for tremolite asbestos fibers; also, the presence of Al was additional evidence against tremolite since it would not be expected to occur in the latter. Asbestos fibers or ferruginous bodies, if present, were below the level of detection of our analysis and

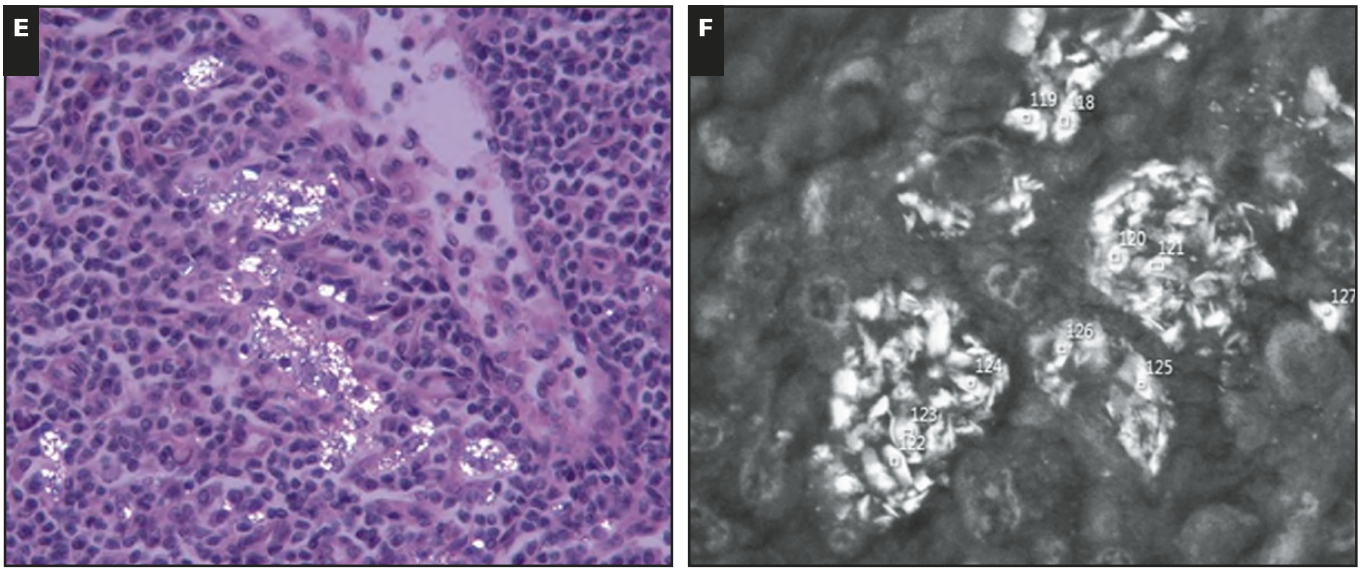

Image 4 (cont) **E**, Same histologic fleld as **D**, under polarized light microscopy, with numerous birefringent particles similar in size and appearance to those seen in the exocervix, within intranodal macrophages (H&E, ×400). **F**, SEM (×500) from the same general area as **D** and **E** but in a different histologic section, showing numerous backscattered electron-positive particulates within the cytoplasm of macrophages, similar to **C**, and the majority having a spectrum characteristic of talc.

hence not found. A representative sampling of the fibers and fiber-like particles we found is shown in **Image 9**, along with more details on the atomic weight percent calculations.

Supplementary Table 1 shows the clinical, light microscopic, and SEM data for the six control patients with no history of perineal or body use talc exposure. Ten ovary blocks and one fallopian tube block comprised the six control patients' materials. Polarizing light microscopy, as shown in the table, revealed a range of two to 17 birefringent particles per slide; these values are comparable to the lower end of the polarizing light microscopy results of the exposed patients in Table 2 but markedly less than for tissues from those patients who had substantial talc by subsequent SEM/EDX. Inflammatory infiltrates, when seen in the control tissues, were generally attributable to the presence of nearby tumor and not to the presence of the uncommon birefringent material. Giant cells, such as were seen in some talc-exposed patients, were not observed in controls.

Correlative SEM/EDX of the control tissue blocks showed a total of four talc particles across all patients: two in patient 2 (right ovary) and two in patient 3 (right fallopian tube). Of note, in Supplementary Table 1, both these patients had pelvic surgery more than 30 years prior to their ovarian cancer surgical procedure. The talc particles represented a very small proportion (0.8%) of the overall backscattered electron-positive particles that were found and analyzed across the 11 control tissue blocks (494). Of those, most were endogenous, the most common being calcium phosphate (202 [41%] particles), sodium salts (108 [22%] particles), and iron phosphate (56 [11%] particles). Nonspecific minerals accounted for 105 (21%) particles; these may access the genitourinary tract through hygiene practices and general living. No fibers, talc or otherwise, were found in any control tissues by SEM/EDX.

Discussion

The cases reported here show in vivo pelvic migration potential for talc that has, to our knowledge, not been reported previously. Within a set of five patient cases, all with known talc exposure to the perineum and all of which had groups of pelvic organs/tissues surgically resected for the management of ovarian carcinoma, talc was found in two pelvic organ sites (three patients), three sites (one patient), and four sites (one patient) distant from the original site of application (perineum). In four of the five patients, pelvic region lymph nodes were one of the sites affected. Talc has been described in one distant pelvic organ site before[15,19] but, prior to this report, not more than one such site in a given patient.

It is important to remember that, because the in situ SEM technique examines only a very small volume of tissue, the finding of even modest numbers of exogenous particles (eg, talc) in tissue sections may translate into a

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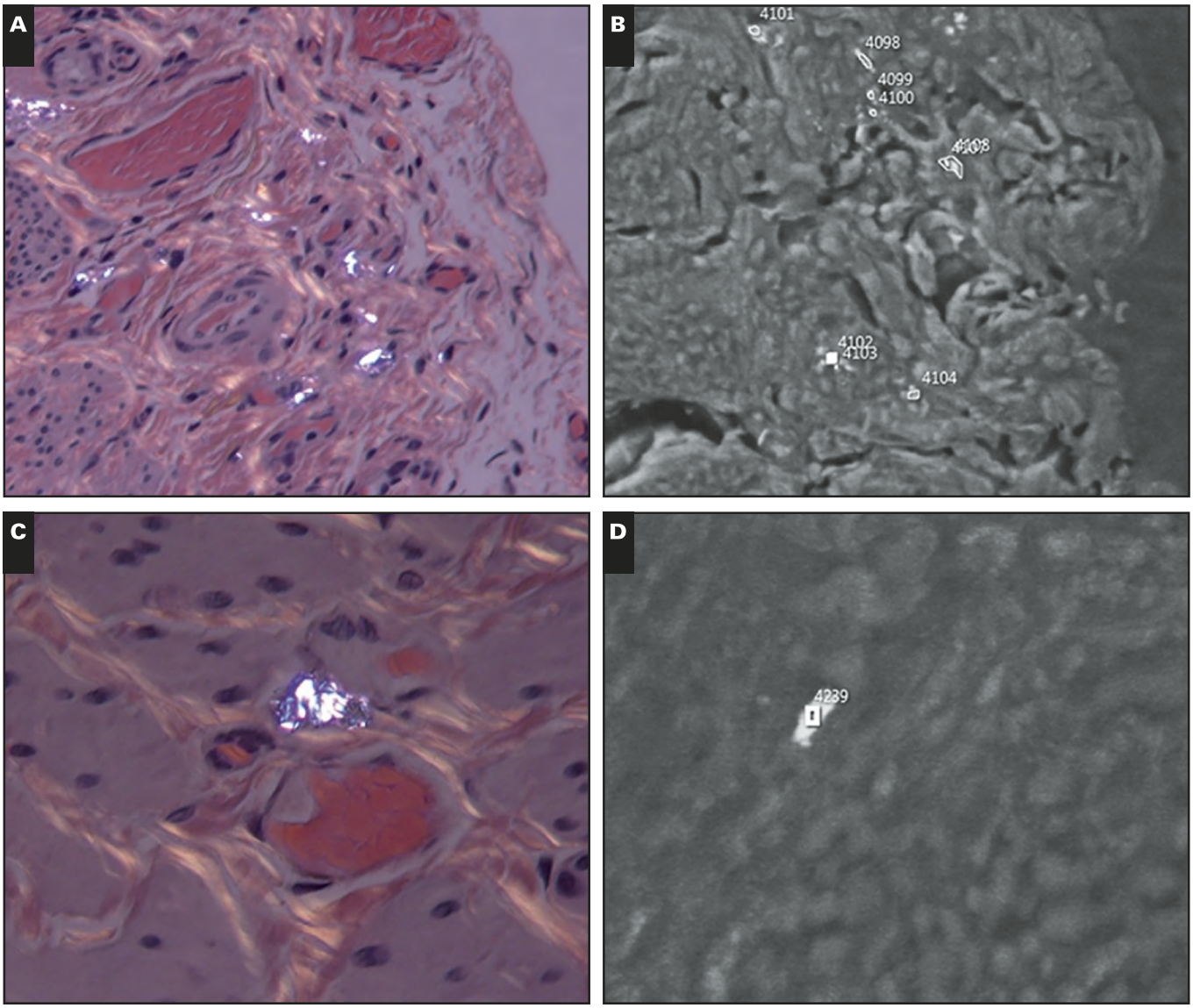

Image 5 Representative photomicrographs for patient 2. **A**, Uterine serosa showing numerous birefringent particles 1 to 10 mm in diameter within soft tissue and macrophages (H&E, ×200). **B**, Scanning electron microscopy (SEM) corresponding to **A**, showing numerous backscattered electron-positive particles (×500), the majority with an energy-dispersive X-ray analysis (EDX) spectrum characteristic of talc. **C**, Fibromuscular soft tissue near fallopian tube, showing a macrophage with abundant intracellular birefringent material similar to that seen in **A** (H&E, ×400). **D**, SEM of the same region as **C**, showing a backscattered electron-positive particle approximately 5 μm in diameter that proved to be talc using EDX (×500).

significant exposure when calculated on a per-gram-of-tissue basis and when placed in appropriate clinical context. Or, to put it another way, seeing particles by in situ microscopy (both light and SEM) requires a relatively large amount of material distributed within the tissues to make it possible to find it in this manner. Roggli and Pratt[25] demonstrated this principle by showing that the identification of one asbestos body in a tissue section corresponded to at least 100 fibers per gram of tissue.

The six control cases supported the contention that talc is rarely found in surgically resected pelvic tissues from patients with no prior perineal or body use exposure. The four talc particles found by SEM/EDX were in two patients who had undergone pelvic surgical procedures more than 30 years prior. Given that history and timeline, the talc could have been introduced from the ambient environment or from talc on instruments or gloves. The latter was relatively common decades ago when these patients had surgery.[26] Birefringent particles of other etiologies (endogenous or nonspecific mineral particles) can be found in nontalc-exposed patient tissues, as was the case in our controls, with SEM/EDX useful in

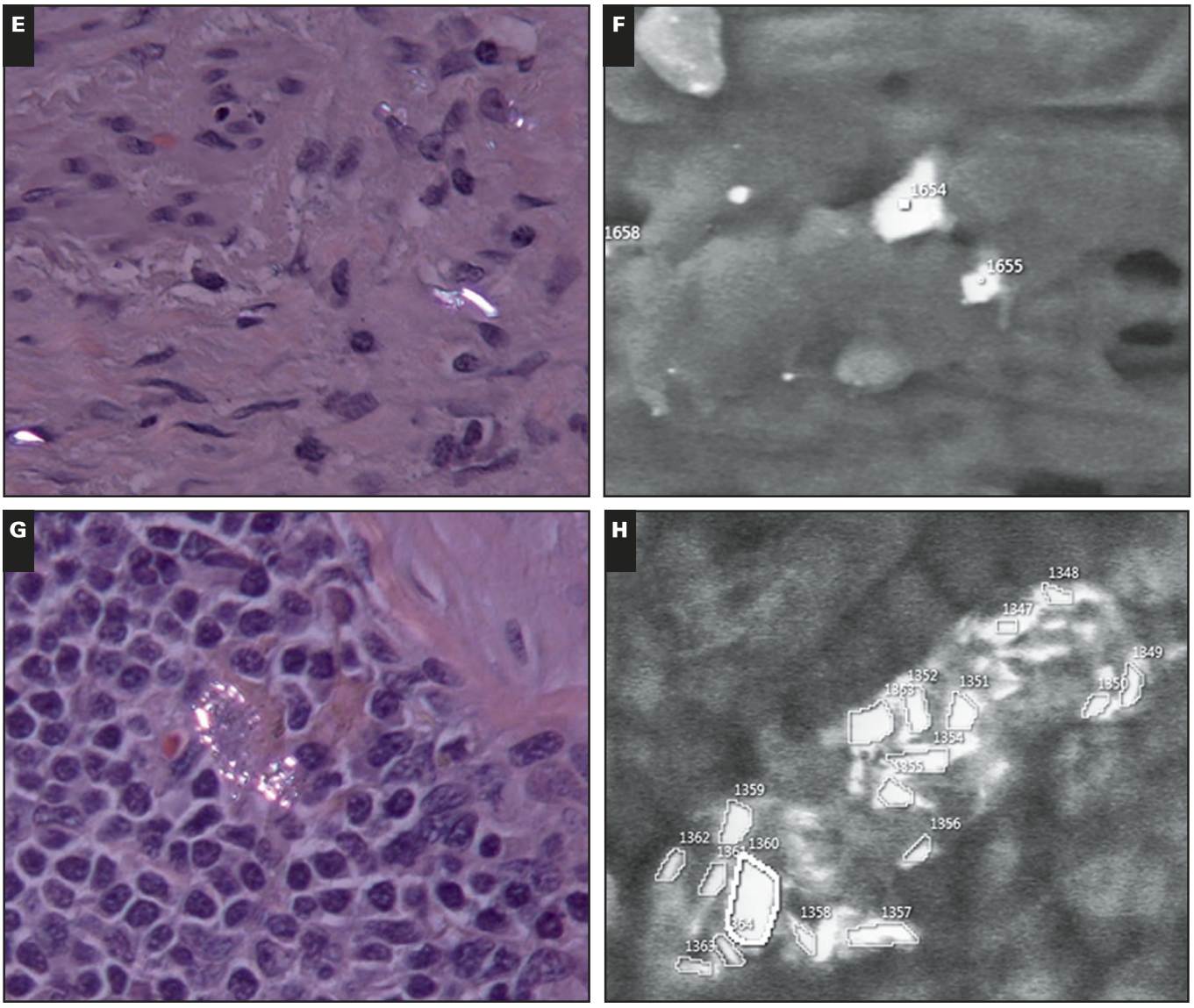

Image 5 (cont) **E**, Birefringent particle seen in soft tissue of ovary, with mixed inflammation and fIbrosis in the general background (H&E, ×400). **F**, SEM showing several backscattered electron-positive particles within the same region as **E** (ovary). The particles were irregularly shaped, less than 7 μm in diameter, and on EDX analysis showed the characteristic spectrum of talc (Image 1) (×500). **G**, Left pelvic lymph node with numerous birefringent particles similar in size and appearance to those seen in the uterine serosa, within intranodal macrophages (H&E, ×400). **H**, SEM from the same general area as **E** but in a different histologic section, showing numerous backscattered electron-positive particulates within the cytoplasm of macrophages, similar to **E**, with most having an EDX spectrum characteristic of talc (×500).

the distinction. However, most of the numerous calcium phosphate particles found in the controls would likely have been nonbirefringent and thus not detected by polarizing light microscopy.

The five cases described here were part of a larger group of cases (all women with ovarian carcinoma and with perineal talc exposure) that were received by us for consultative purposes over a 3- to 4-year period. Among 34 consults recently reviewed by one author (S.A.M.), 29 (85%) had birefringent particles in more than one pelvic organ site, and of the five that did not, three had substantially limited material for review. Most of these cases have not yet had SEM/EDX performed on tissue blocks, so we do not yet know to what extent these light microscopic findings translate into sites of talc migration. But these preliminary data suggest that a substantial fraction might among patients with the appropriate exposure history.

A prominent finding in several of our cases and tissue sites was the accumulation of numerous birefringent particles in the cytoplasm of tissue macrophages

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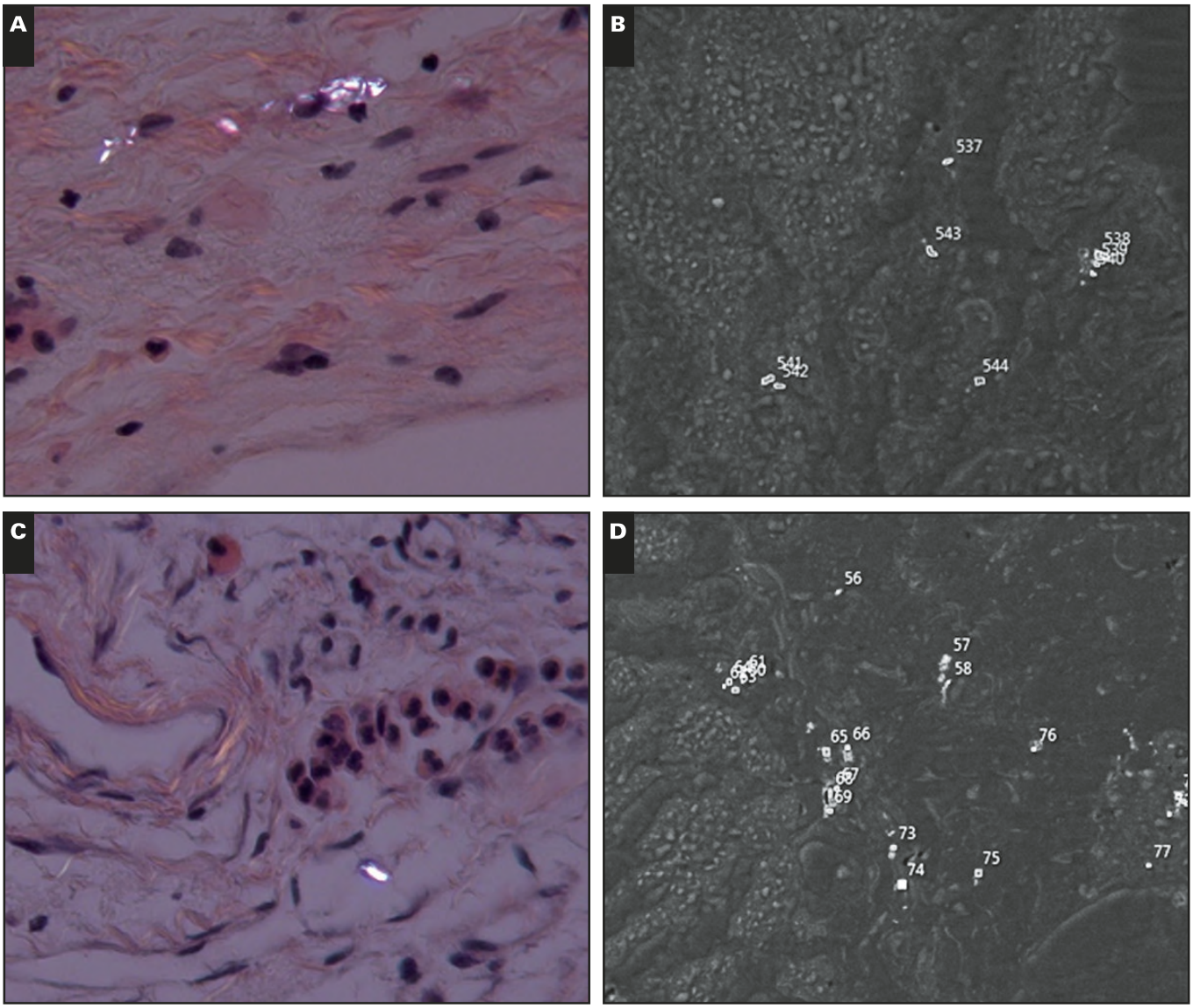

Image 6 Representative photomicrographs for patient 3. **A**, Uterine serosa showing numerous birefringent particles 1 to 10 μm in diameter within soft tissue and macrophages (H&E, ×400). **B**, Scanning electron microscopy (SEM) corresponding to **A**, showing numerous backscattered electron-positive particles (×500), most with an energy-dispersive X-ray analysis spectrum of talc similar to Image 1. **C**, Ovarian stroma showing a birefringent particle within soft tissue but close to blood vessel. Other birefringent particles were seen in different microscopic flelds in this section. **D**, SEM showing several backscattered electron-positive particles within the same tissue (but different section) corresponding to **C**. These backscattered electron-positive particles showed a spectrum characteristic of talc (Image 1) (×500).

on both light microscopy and SEM, which, using EDX analysis, proved to be talc. The H&E appearance of these macrophages was often rather subtle, with grayish cytoplasm and a faintly ground-glass appearance; in our opinion, they could be easily missed on a routine slide review where just light microscopy is performed. This therefore highlights the importance of doing polarizing light microscopy on surgically resected pelvic tissues, not necessarily in every case but indeed if or when the appropriate talc exposure history is present.

Talc is able to stimulate the phagocytic potential of macrophages: a subset of the current authors and their colleagues reanalyzed the slides from the study by Beck et al,[27] who did in vivo hamster studies using sonicated intratracheally induced talc and granite exposure. It was found that pulmonary macrophages phagocytize talc more avidly than granite, especially in the initial 1 to 2 days following exposure (unpublished data). Beck et al[27] showed that these macrophages that have ingested talc are then unable to phagocytose radiolabeled particles

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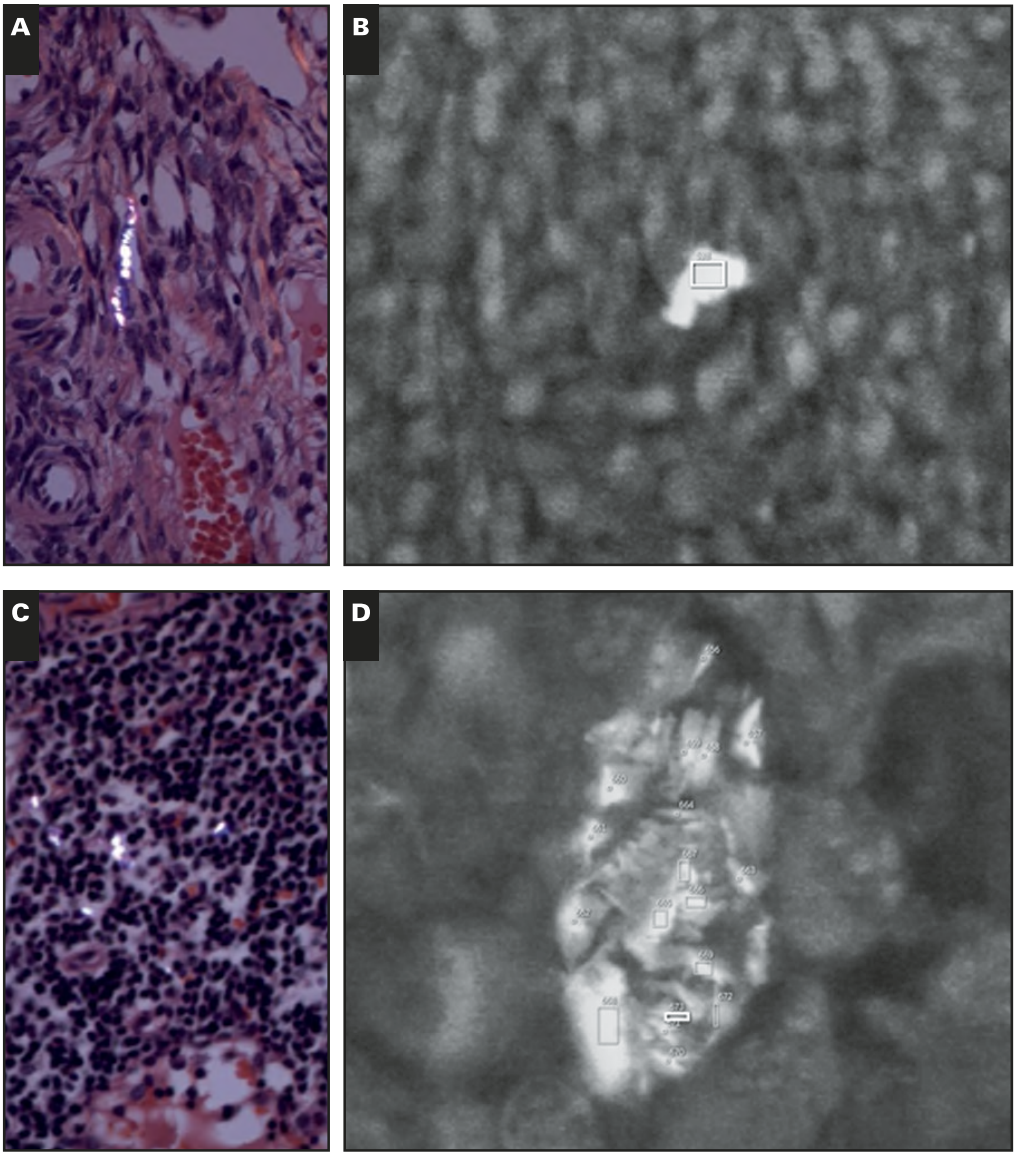

Image 7 Representative photomicrograph for patient 4. **A**, Tissue of the right ovary with a string-like arrangement of multiple birefringent particles (1-5 μm in greatest dimension) within ovarian stromal tissue (H&E, ×400). **B**, Scanning electron microscopy (SEM) showing an irregularly shaped particle, which in backscatter mode is about 6 μm in diameter. Energy-dispersive X-ray analysis of this particle showed the typical spectrum of talc (Image 1) (×1,000). **C**, Right pelvic lymph node tissue with approximately eight birefringent particles (each ~2 μm in greatest dimension or less) visible in the same plane of focus with the cells of the lymph node. Many of these particles are clearly within macrophage cells in the lymph node. **D**, SEM of the same right pelvic lymph node tissue (but a different section) showing numerous backscattered electron-positive particles within the cytoplasm of a macrophage, similar to the light microscopic morphology in **C**. These particles had the characteristic spectrum of talc (Image 1) (×1,000).

as readily as macrophages that have ingested granite or control macrophages, which may be why we also observe talc particles in tissue outside of phagocytic cells. This apparent initial avidity of macrophages for talc is consistent with the morphologic findings in our case series and may help explain the inflammatory potential of talc. Full reviews of macrophage biology and inflammatory responses are available in the literature, including the phenomena of reactive oxygen species generation and opsonization.[28-31] Talc may remain long after the initial inflammatory response has run its course, as evidenced by studies showing that talc has a slow dissolution rate in tissue.[24] In addition to the macrophage activity described earlier, mixed inflammatory infiltrates were sometimes seen in our talc-containing cases, for example, in the fallopian tube in patient 5 (Image 6) and the ovary in patient

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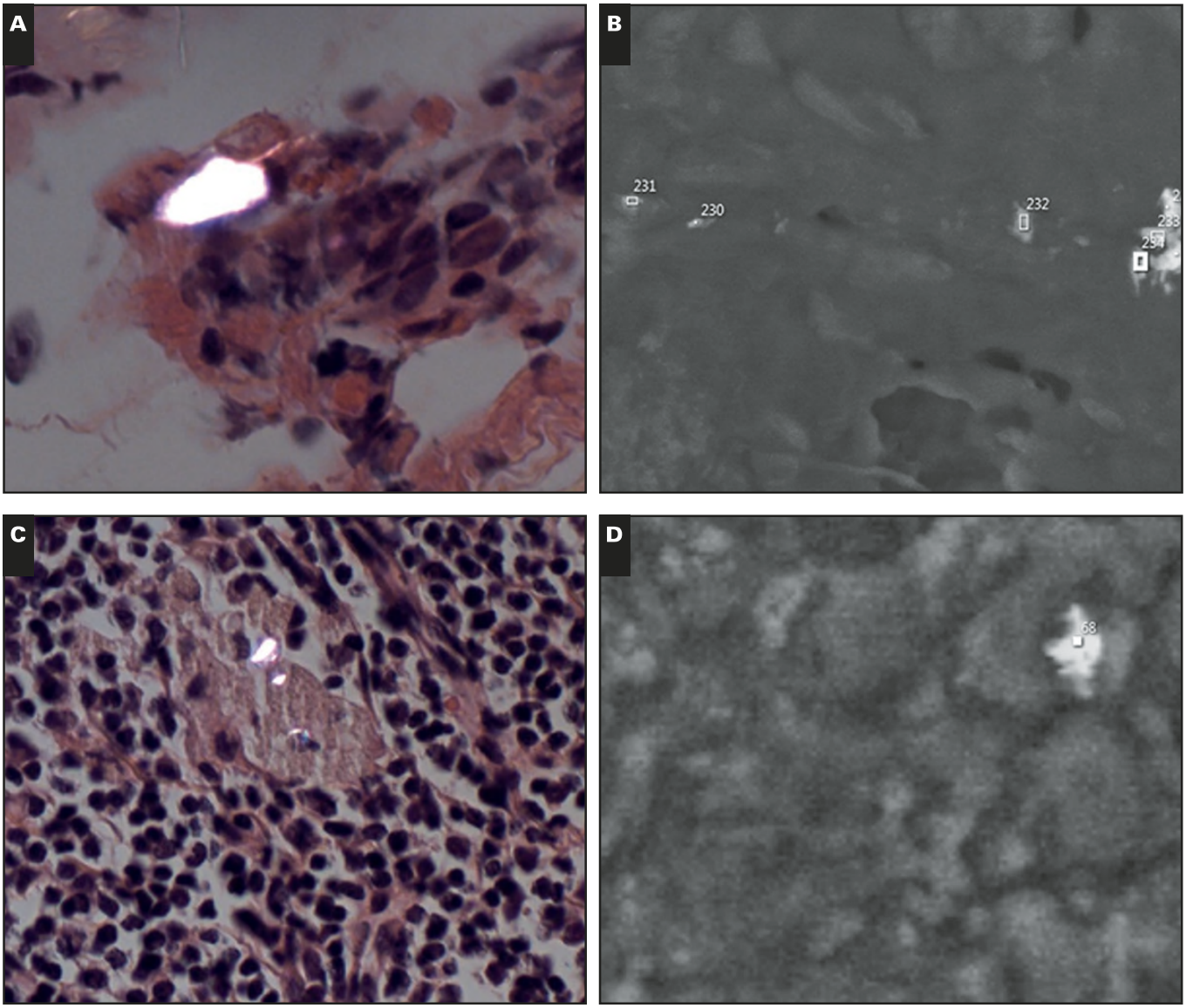

Image 8 Representative photomicrograph for patient 5. **A**, Birefringent particle approximately 7 μm in diameter, found in the soft tissues around the fallopian tube, and associated with chronic inflammation (H&E, ×400). **B**, Scanning electron microscopy (SEM) showing numerous backscattered electron-positive particles within the soft tissues around the same fallopian tube tissue as in **A** but a different tissue section (×500). **C**, Two birefringent particles within a left pelvic lymph node (H&E, ×400). **D**, SEM of the same pelvic lymph node tissue as in **C**, showing an irregularly shaped, backscattered electron-positive particle less than 5 μm in diameter, which showed the characteristic spectrum of talc.

2 (Image 3). The understanding of talc's ability to induce inflammation is well established.[32]

Through the migration of particles to lymph nodes as well as to other pelvic sites, the morphologic findings in our study indicate the likely importance of lymphatic pathways in the migration of talc. Talc may access lymphatics directly in the perineum (its typical initial exposure location) or at any point in its ascent through the genitourinary tract toward the fallopian tubes and ovaries. Among other possible mechanisms, this might occur through erosions in the superficial epithelial surface, thereby exposing the lymphatic channels directly underneath. Once talc particles reach ovaries and/or pelvic region lymph nodes, they have access to a further network of lymphatics present in those locations, thus yielding further migration potential. One example would be talc migration to para-aortic nodes, which we have seen in one patient (not included as part of this series but included in a separate report[19]) and conceptually mirrors the clinical finding that ovarian serous carcinoma tends to

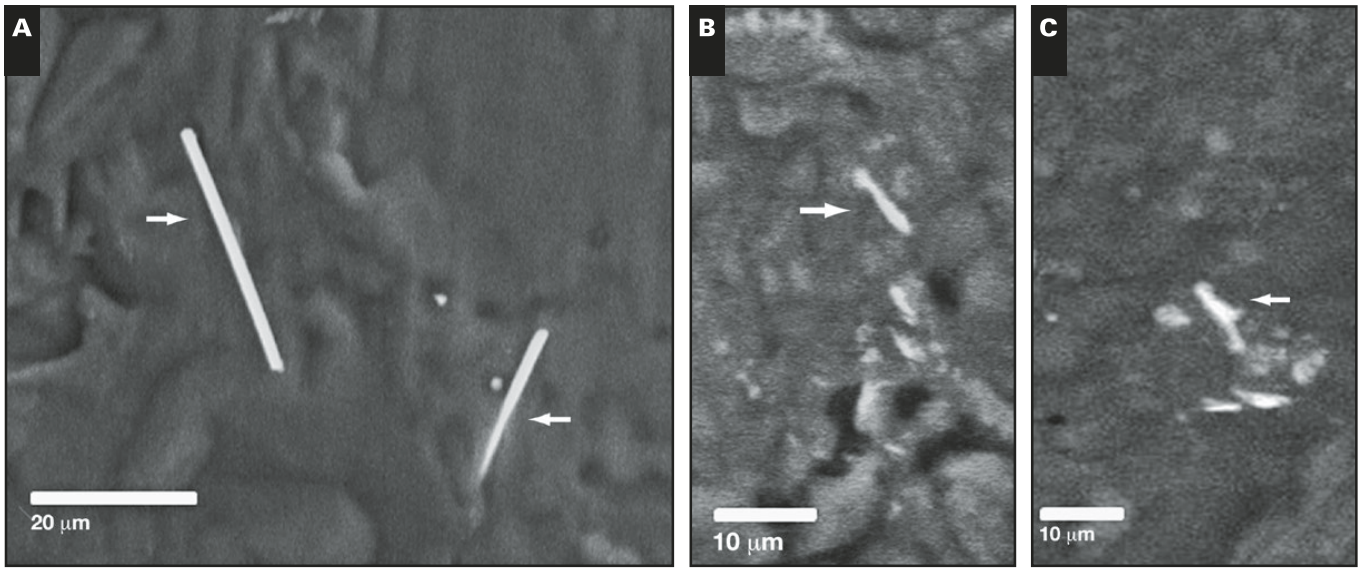

Image 9 Representative fibers and fiber-like particles (≥5:1 aspect ratio) found in our patient analysis (all photos are scanning electron microscopy with backscattered electron imaging, ×500). **A**, Long aspect ratio fibers (arrows) with a chemical signature of calcium (Ca), magnesium (Mg), aluminum (Al), iron, and silicon (Si) found in patient case 2 (right ovary). Fiber at right is seen to be extending into tissue where it disappears from view; thus, its aspect ratio may be higher than what is visible. For the total of four long aspect ratio fibers (10:1 or greater) that we found in our study, based on atomic weight percent calculations, average Mg/Si was 0.241 and Ca/Si was 1.03, where the respective ratios expected for tremolite are 0.542 and 0.357. Average Al/Si for the four fibers was 0.327, whereas no Al is expected for tremolite. **B**, Talc fiber-like particle (arrow) with an approximately 6:1 aspect ratio from case 2 (uterus). **C**, Talc fiber-like particle (arrow) from case 3 (uterus), with an approximately 6:1 aspect ratio. Particles in **B** and **C** had Mg-Si spectra with atomic weight percent ratios within 5% of the theoretical value of 0.649 for talc, similar to Image 1. In **C**, the other nearby backscattered electron-positive particles were also talc but did not meet the 5:1 aspect ratio threshold for a fibrous morphology.

metastasize early to para-aortic nodes preferentially over other node groups.[33] Theoretically, talc could even reach distant extrapelvic sites through further lymphatic spread and induce inflammatory reactions there, but in the women with ovarian malignancy who we have evaluated, this type of study opportunity has not arisen, simply because these extrapelvic tissues do not become available for examination as part of TAH/BSO surgery.

Besides the finding (with obvious implications) of exogenous material in lymph nodes, in our set of cases, evidence of lymphatic migration was seen in the distribution of birefringent material around small blood vessels in the uterine serosa (cases 2 and 3) and soft tissues at the periphery of the fallopian tube (cases 2 and 6). These areas are rich in lymphatics, and the clustering of exogenous material there is strongly suggestive of migration via such a route. Lymphatic vessels are highly distensible and compliant and have an elaborate pumping mechanism consisting of contractile lymphatic muscle cells and one-way valves that facilitate the transport of material (whether endogenous or exogenous) consistently via an anterograde flow route.[34] Initial lymphatics present in peripheral locations, such as those likely to be encountered in talc exposure, are typically tens of microns in diameter,[34] well within the range of the 1- to 10-μm size typically seen for talc particles in exposure settings and also consistent with the size of pathogens, malignant cells, and other materials the lymph system typically collects and transports.

Sentinel lymph node studies, although derived from oncology, offer insight into the migration potential of talc from the perineum or lower genital tract and help explain the peculiar idiosyncratic specificity of talc migration sites that is often seen in patient cases. The general principle from sentinel lymph node studies is that usually there is one node or at most a small group of nodes that represent the initial site of dissemination or metastasis in a given patient, among many nodes that in theory are part of the drainage basin,[35] and so if that sentinel node is free of metastases, then remaining nodes in the same nodal basin should be free also.[36] The lymphatic network of the ovary is known to be both rich and complex and subject to frequent remodeling based on the menstrual/hormonal cycle.[37] Based on studies of ovarian

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malignancy, the most common drainage sites from the ovary are the pelvic, paraaortic, and iliac lymph nodes,[38] so talc that migrates to the ovary would then be expected to have access to these lymph node groups through a similar mechanism. A further study by Vanneuville et al[39] using lymphoscintigraphy on 14 patients showed that ovarian lymphatic drainage may be age dependent, with premenopausal drainage likely to be both pelvic and para-aortic but postmenopausal drainage likely to be predominantly para-aortic. As for the lymphatic drainage basin for the uterus, pelvic and para-aortic nodes may become involved simultaneously, in contrast to those in the cervix, where pelvic nodes become involved first and then result in spread to para-aortic nodes.[40] Further sentinel lymph node studies have showed that within the uterus, upper and lower drainage pathways exist, with the former draining to external iliac and obturator lymph nodes and the latter draining to the internal iliac and presacral lymph nodes.[41] Furthermore, pelvic lymphatic pathways are frequently anastomosing, idiosyncratic, and subject to modification.[37,39,40]

Taking all of this together, it seems prudent to conclude that once talc gains access to the lymphatic system anywhere in the female genital tract, it could potentially be detected in any of the lymph node groups previously described for which metastases and sentinel lymph node tracers have been localized in the past, as well as in solid organ sites (ovary, fallopian tube, uterus), which contain efferent from those same lymphatic networks. This is entirely consistent with the spectrum of histologic findings that we report in this case series. It is also likely that patterns of talc dissemination, like patterns of lymphatic drainage and metastasis in other settings, are likely to be idiosyncratic and patient dependent, without clear explanations in most cases as to why a given patient localized foreign material in a particular node or site and not other sites, other than a given particular drainage pathway simply being what that patient's anatomy prefers. Other factors, such as the overall burden of exposure, the exact sites of exposure and the nature of the physical application, and the size distribution of the talc particle exposure, all also likely play roles in whether and where pelvic migration pathways develop.

Among the five patients in the main study, two had a history of tubal ligation (cases 2 and 3, Table 1). In theory, this should reduce the risk of ovarian carcinoma from talc exposure by blocking the latter's ascension to the ovaries through the reproductive tract, thus mitigating inflammatory effects. In fact, some but not all studies have shown an increased risk in malignancy from talc use in women who have not undergone tubal ligation.[5,42,43] What is interesting is that the two patients in our study with tubal ligations had numerous talc particles in a strongly lymphovascular distribution in their uterine serosal areas (Tables 2 and 3 and Image 3), with patient 2 also showing abundant talc in pelvic region lymph nodes (nodes were not sampled in patient 3). Although the numbers here are too small to draw definitive conclusions, an interesting possibility is that blockage of the reproductive tract passage may lead to countervailing greater access of talc to the lymphatic system, especially if exposure levels are high.

Talc found in our study was usually polygonal and nonfibrous; nevertheless, 18 fiber-like talc particles were found across the main part of the study, with an aspect ratio of 5:1 or more. These were typically found in areas with large collections of talc particles overall (eg, macrophages, lymph nodes) and so most likely simply represent one end of the size distribution of naturally heterogeneous particles in size and shape. Only four long aspect fibers (≥10:1) were found, and these were nonasbestos.

The expanded understanding of talc's biologic potential, as evident in this set of cases, has implications for surgical pathologists who review TAH/BSO specimens from patients with ovarian carcinoma. If a history of talc use is known or suspected, it may be prudent to examine with polarizing light microscopy the range of tissue types studied in this case and not simply the ovaries (although the latter is indeed a prudent place to start, especially if benign residual stroma can be found). If birefringent particles are identified in the tissue, the corresponding slides and blocks can then be referred for SEM/EDX analysis for confirmation. Based on our surgical pathology experience, the macrophage, giant cell, and chronic inflammatory infiltrates seen in some of our talc-containing cases by light microscopy are unlikely to be pathognomonic alone. Thus, while their presence is of interest, especially in the right clinical setting (ie, history of talc use), the auxiliary studies described here would be needed. Our concomitant study of six patient controls supports the contention that talc is rarely found in the pelvic tissues of nonexposed patients. The findings in the main study, especially balanced against the control tissue findings, further support the contention that unexpected or unexplained inflammatory infiltrates (especially chronic or macrophage-rich), combined with birefringent material on polarized microscopy, should prompt SEM/EDX for confirmation of talc (or, if not talc, whatever the exogenous substance may be).

Conclusion

The existence of morphologically demonstrated talc in multiple pelvic organ sites, including pelvic tissues and lymph nodes simultaneously, which is reported here in

multiple patients, has not been reported to our knowledge. Given the ongoing concerns regarding talc, particularly with regard to its epidemiologic association with ovarian cancer, these findings are important and offer new insight into the biologic potential of talc, its inflammatory potential, and its migration via pelvic lymphatics from the perineum. Along with the available epidemiologic studies and the few previous morphology-based reports, the findings suggest that clinicians may want to closely examine pelvic organs and lymph nodes (when made available through surgical resection) for talc in patients with ovarian carcinoma and a history of perineal talc use. The index of suspicion is especially high in cases with birefringent material (by polarizing light microscopy) and unexplained chronic or macrophage-rich inflammatory infiltrates in pelvic tissues.

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