Objective: Many high-grade serous ovarian carcinomas are thought to originate from fallopian tube epithelium. Karyometry detects chromatin abnormalities at the nuclear level using high-resolution computer imaging analyses. This study hypothesizes that karyometry can detect nuclear abnormalities of fallopian tube epithelium in women at high risk as compared to low risk for ovarian cancer.

Study Design: Fallopian tube tissue from 8 women carrying BRCA1 or BRCA2 alterations (high risk) and 7 women at normal risk were obtained from the tissue bank at NorthShore University HealthSystem. Tissues were fixed, paraffin embedded, sectioned, and stained, followed by high-resolution imaging and karyometric analysis.

Results: The distribution of nuclear features and nuclear signatures in tubes from women at high risk showed a distinct deviation from that of normal risk cases. The two most segregating features in the discriminant function scores (pixel optical density heterogeneity...
and the number of very dark stained pixels) showed a pronounced shift from normal in high-risk nuclei and represent a statistically significant difference (p<0.05) at the nuclear level.

CONCLUSION: Karyometry detected an abnormal morphometric phenotype in nuclei of fallopian tube epithelium from women at high risk for disease as compared to normal controls. (Anal Quant Cytopathol Histopathol 2021;43:44–51)

Keywords: BRCA1 genes, BRCA2 genes, carcinoma, fallopian tube, karyometry, karyometric image analysis, ovarian cancer, ovarian epithelial carcinoma.

Ovarian cancer is the most lethal gynecological malignancy in women and is responsible for 5% of all cancer-related deaths in females.1 When detected in early stages, the 5-year survival rate is as high as 90%.1 Unfortunately, only 15% of cases are diagnosed at this stage, and ovarian cancer typically progresses to later, more aggressive stages before detection in the majority of cases, thereby resulting in a substantial drop in 5-year survival to less than 40%.1

Women with mutations in breast cancer susceptibility genes, BRCA1 and BRCA2, have a 40% and 18% lifetime ovarian cancer risk, respectively.2 For these women, twice yearly screening has been advised in the hope that ovarian cancers might be detected early. Current screening techniques including pelvic examination, transvaginal ultrasound (TVU), and CA125 blood marker measurement, however, have very poor specificity and sensitivity.3 Furthermore, annual screening by TVU and CA125 in high-risk populations is grossly inefficient at detecting the disease in its early stages.4 Because of the limitations in ovarian cancer screening, it is recommended that women at high risk for the disease undergo radical preventative measures, such as a prophylactic bilateral salpingo-oophorectomy (BSO), when they have either completed childbearing or by age 35–45.5

Bilateral salpingo-oophorectomy, or the removal of both ovaries and fallopian tubes, has been associated with an 80% reduction in not only ovarian cancer risk, but also fallopian tube and peritoneal cancer risk.5 Although removal of the ovaries and fallopian tubes is an effective method of ovarian cancer prevention, it is accompanied by adverse sequelae, including loss of fertility, sudden onset of menopause, and rapid loss of bone mineralization.6 In order to decrease the need for such harmful surgeries, more efficient screening techniques are required to accurately identify individuals harboring lesions at risk for malignant transformation and to use these techniques to detect the disease in its early, more curable stages.

Karyometry is a form of digital microscopy that allows for quantitative analysis of diagnostic images. All digital images are represented by discrete points or picture elements known as pixels. Electronically examining images on a pixel-by-pixel basis offers novel, objective information and descriptive features that are not readily visible to the human eye. The difference between visual and computed features can be demonstrated by reading printed text. Although one can recognize visual information like letters, words, and language, more complex information, such as the relative frequency of occurrence of one letter compared to another, is overlooked. The relative frequencies of certain letters or vowels compared to others, however, characterize the text while providing objective and computed descriptive features. Similarly, examining the two-dimensional spatial and statistical distribution of pixels in a digitized image produces computed descriptive features. Through the power of variance-analytic procedures, numerical representations of these features allow for subtle, yet consistent, differences to be identified between images. The computer processing of digitally represented histopathologic images provides the unique opportunity to collect quantitative, numerical data from an otherwise qualitative source.

Karyometry can ascertain chromatin abnormalities at the nuclear level by utilizing high-resolution computer imaging analyses. It is designed to extract karyometric features that are undetectable to the human eye, thereby providing a level of sensitivity that allows the fine detection of variability between samples despite the natural heterogeneity of nuclei.7 Not only does karyometry have the ability to accurately detect nuclear abnormalities, it also has provided objective and statistically valid measures of chemopreventive efficacy in vivo. For example, a statistically significant change toward normalization in the karyometric features in the ovary and fallopian tube epithelium in women at increased risk for ovarian cancer treated with the progestin levonorgestrel supported the notion that progestins may act as chemopreventive agents against ovarian and fallopian tube cancer.8 Similar
studies investigating chemopreventive efficacy of vitamin A in sun-damaged skin have shown that karyometry analysis can provide more objective measures of chemopreventive efficacy.9,10

When pathologists microscopically examine tumor biopsies, they generally assign a grade based on how abnormal the cells appear to visual inspection. On the other hand, digital analysis of biopsy images through karyometry can provide the ability to compute and document certain features that are too small to be appreciated by visual observation. For example, a previous study using karyometry on ovarian surface epithelium discovered that histologically normal-appearing epithelium in women at high risk for ovarian cancer, as well as in the normal epithelium adjacent to ovarian cancer, harbors abnormal submicroscopic changes in the nuclear chromatin, thus suggesting potential for malignancy.11 Furthermore, studies utilizing karyometry have uncovered similar significant relationships between slight changes in the nuclei of normal, preinvasive, and invasive lesions in various other tissues including breast, rectal, urinary tract, prostate and skin.11-16 The ability to detect distinct changes in nuclear chromatin pattern between normal, preinvasive, and invasive lesions provides the opportunity to characterize samples in a progression curve. In fact, karyometry has been shown to accurately detect the progression of precancerous lesions of actinic keratosis to aggressive squamous cell carcinomas through the comparison of karyometric features.17 Although further research is required, karyometry possesses the potential of being an instrumental screening technique for cancers of many different origins.

It is a widely supported theory that the cell of origin for many high-grade ovarian cancers may originate from the fallopian tube epithelium rather than ovarian epithelium.18 This makes the fallopian tube an attractive target for ovarian cancer screening and prevention. In the current study, we sought to determine whether karyometry might be useful in detecting nuclear abnormalities of the fallopian tube epithelium in women carrying the BRCA1 or BRCA2 mutation at high risk for ovarian cancer as compared to women with normal risk factors. The ability to evaluate nuclear abnormalities of fallopian tube epithelium through karyometry may provide a more accurate assessment of ovarian cancer risk beyond a simple genetic test for BRCA mutations. Therefore, karyometry could result in more individualized assessment of disease risk and the avoidance of unnecessary and harmful procedures. It may even lead to the detection ofpreneoplastic lesions, resulting in appropriate preventative measures and early surgical intervention and, in this way, become a useful element of precision oncology.

Materials and Methods
Sample Collection
A portion of fallopian tubes included in this retrospective study were collected from an archive of de-identified samples and received exemptions from the Human Subjects Review Committees at the University of Arizona and NorthShore University HealthSystem in Chicago. The remaining samples were collected under IRB protocol, utilizing the U.S. common rule and obtaining patient consent when appropriate. All materials were transferred to the University of Arizona from the Pathology Department at NorthShore University HealthSystem (Evanston Hospital) on the basis of a material transfer agreement between these two institutions.

The “normal risk” fallopian tubes were collected from women who lacked BRCA1 or BRCA2 mutations or a strong family history of breast and ovarian cancer and underwent a salpingectomy for benign gynecologic indications. The “high risk” fallopian tubes were collected from women who were positive for BRCA1 or BRCA2 mutations and who underwent surgery for ovarian cancer risk reduction. All high-risk cases were examined carefully by pathologists to rule out the presence of any neoplasia, including the presence of atypia, STIC lesions, and cancers. They were therefore confirmed to be histologically normal, and any abnormality detected by karyometry was therefore occurring prior to any histologic transformation.

Tissue Preparation
A total of 15 fallopian tubes were collected, consisting of 8 tubes from women at high risk due to BRCA1 and BRCA2 gene mutations and 7 tubes from women at normal risk. Tissue fixation and staining was strictly controlled to prevent unnecessary sources of variability. Tissues underwent standard fixation in 10% neutral buffered formalin, followed by paraffin embedding. They were then sectioned at 5-micron thickness and stained with a reproducible procedure using synthetic hematoxylin and eosin. Human tonsil tissue was utilized in order to maintain quality control during the
staining procedure. Images of the fallopian tube epithelium were captured at high magnification (100:1) with a high-quality video microscope, utilizing a high numerical aperture (1.40) apochromatic oil immersion objective. Images were recorded at 4 to 6 pixels per micron and consisted of a random accumulation of well-defined nuclei that did not overlap.

**Karyometric Protocol**

Images were analyzed using a semi-automated segmentation program with manual correction, thereby isolating individual nuclei for further investigation. Segmentation of nuclei was a crucial step in quantitative image analysis because any error in defining nuclear borders would have modified the values utilized by karyometric features. For segmentation, an image-processing algorithm was applied for object recognition. This resulted in chain codes that outlined each individual nucleus, thereby specifying the pixels that generated the nuclear border. Interactive corrections were required throughout the segmentation process since a single algorithm rarely segments all nuclei correctly. This program isolated, on average, 158 nuclei per fallopian tube for a total of 1,257 nuclei in the high-risk group. Likewise, an average of 172 nuclei per fallopian tube for a total of 1,205 nuclei were isolated in the normal-risk group. Figure 1 provides a sample representative image of the fallopian tube epithelium after nuclear segmentation.

Following segmentation, 93 karyometric features were used to analyze nuclear chromatin pattern. Global features are the simplest and utilized all pixels localized in a single nucleus, including such characteristics as “nuclear area,” “nucleocyttoplasmic ratio,” “measures of roundness of a nucleus,” and “total optical absorbance.” These features represent individual nuclei as a whole and can be classified as zero-order relationships. First- and second-order features investigate the differences or similarities between adjacent pixels, thereby analyzing the 2-dimensional relationships of contiguous pixels to determine chromatin patterns. For example, several features summarize chromatin pattern by utilizing “pixel run length” or “mean pixel absorbance.” Finally, the associations of several nuclei throughout a sample are organized into third-order relationships. Essentially, the features classified in higher-ordered groups are associated with more complex linear combinations.

A more descriptive list of the 93 features has been published previously.

**Statistical Analysis**

The 93 karyometric features collectively created the “nuclear signature,” and data were normalized to provide comparable data sets. Furthermore, “nuclear abnormality” was calculated by averaging the standard deviations for all 93 features of each nucleus. The nuclear signature expresses the values and correlations among all measured karyometric features, thereby providing a quantitative approach that can discriminate malignancy-associated changes in the nuclear chromatin from benign. When using karyometric features to detect nuclear abnormalities in a tissue biopsy, values are normalized to a reference data set and expressed in units of standard deviation, or z-value. The magnitude of z-value for each feature measures its deviation from a normal reference set. Normalized values add stability and provide data that can be compared to similar samples. The z-values averaged over all 93 karyometric features offer a measure of “nuclear abnormality.”

In many cases the nuclear abnormality value is not particularly sensitive, given that not all 93 karyometric features change between samples. A Kruskal-Wallis test was utilized to ascertain the

![Figure 1](image)

*Figure 1* The representative image illustrates nuclei of fallopian tube epithelium that were randomly selected for further analysis. Images of the fallopian tube epithelium were captured at high magnification (100:1) with a high-quality video microscope utilizing a high numerical aperture (1.40) apochromatic oil immersion objective. The white outlines represent chain codes that defined the border of each nucleus during segmentation.
significance of very small deviations in data sets in order to detect the submicroscopic differences that cannot be appreciated by visual analysis alone. Since there are 93 statistical tests being performed, it is necessary to apply a multiple-test correction such as the Bonferroni correction and the Benjamini-Hochberg procedure to control for false positives.\(^\text{21,22}\) In this work we selected features with statistically significant value differences, at \(p\) values \(<0.0001\), which allows for a Bonferroni correction. This test identified several important features with statistically significant differences between the high-risk group and the normal-risk group.

These karyometric features identified by the Kruskal-Wallis test were then used to construct the linear discriminant function for separating the two groups.\(^\text{20}\) A discriminant function analysis is widely used in problems of categorizing observations into different groups, often called classification problems in statistics. Using the selected features, a discriminant function analysis builds a classification rule—in this case, high risk and normal risk—by developing a function that can separate different groups. Descriptive statistics can characterize a set of nuclei by the numeric values of their features. Finally, Wilks’ lambda was used to determine a difference between the nuclear abnormality in high-risk and normal-risk fallopian tube samples with \(p\) value \(<0.05\).

**Results**

Figure 2 plots the nuclear signature of the samples, i.e., the standardized scores of 93 features, for normal-risk fallopian tube epithelium (top panel) and for high-risk fallopian tube epithelium (bottom panel). It shows that the nuclear feature distribution of high-risk cases demonstrates a distinct deviation from that of normal-risk cases. Wilks’ lambda was reduced to 0.63, thereby providing evidence of a definite difference between high-risk and normal-risk fallopian tube features. Discriminant function analysis highlighted two of the most segregating karyometric features, including pixel optical density heterogeneity and the number of very-dark-stained pixels. The feature “pixel optical density heterogeneity” measured the degree of diversity in the pixel optical density, and the feature “number of very-dark-stained pixels” measured the total number of dark-stained pixels in the nucleus. Figure 3 displays the distribution of discriminant function scores for nuclei from

**Figure 2**

The graphs represent the nuclear signatures for both high-risk (bottom) and normal-risk (top) groups. Each bar represents a single karyometric feature and provides a quantitative value for the 93 global features selected in the karyometric analysis. In other words, each bar represents a \(z\)-value that was calculated using the absolute difference of the corresponding reference feature mean divided by the corresponding standard deviation for the reference feature. There are marked abnormalities in at-risk from normal, as seen by the variation in \(z\)-values.
high-risk cases (in black) and for nuclei from normal-risk cases (in grey). It clearly shows that the discriminant function for high-risk nuclei displayed a pronounced shift ($p<0.05$) away from that of normal-risk cases, towards lower (more negative) values. Additionally, utilizing these two segregating karyometric features in a bivariate plot with 95% ellipses for the case mean values demonstrated a statistically significant difference ($p<0.05$) between the two experimental groups at the nuclear level (Figure 4). It was observed that nuclei from high-risk tissues, falling onto the upper portion of Figure 4, had lower values of the pixel optical density heterogeneity than those from normal-risk tissues.

**Discussion**

This small, exploratory study was designed to assess whether or not karyometric analysis could detect changes in the nuclear chromatin pattern of fallopian tube epithelium from women at high risk for ovarian cancer. The results suggest that there were, in fact, detectable and significant changes in the chromatin signature of tubes in the high-risk group as compared to the normal-risk controls. As illustrated in Figure 2, there was a distinct difference in the nuclear signature of fallopian tube epithelium of high-risk samples as compared to normal controls. Changes in the nuclear signature could represent the earliest transformation of histologically normal-appearing tissue to premalignant lesions. Previous studies confirmed that seemingly normal tissue might harbor premalignant lesions that can be detected through nuclear karyometry. For example, research conducted by Anderson et al uncovered significant differences between nuclei in normal-appearing breast tissue containing remote cancer as compared to nuclei in breast tissue with no cancer. These differences indicate a deviation from normal, thereby suggesting the potential to progress to malignancy.

Pixel optical density heterogeneity and the number of very-dark-stained pixels were computed as the most segregated karyometric features. In fact, these features provided statistically significant evidence that nuclear chromatin patterns in high-risk women differed from those in normal-risk controls (Figure 4). When cells undergo molecular changes, they develop altered chromatin structure. By utilizing karyometric nuclear analysis, it is possible to characterize chromatin patterns by describing the organization of pixel combinations.

It is widely appreciated that BRCA1 or BRCA2 mutations predispose women to high-grade serous ovarian cancer and that the fallopian tubes are believed to be the origin of disease. Therefore, abnormalities between the nuclear signatures of fallopian tubes from women with these mutations and normal controls were anticipated. Given the preliminary nature of this study, one of the weaknesses is the small number of samples analyzed. In the future, we hope to expand upon this research and analyze, in a blinded fashion, a much larger number of fallopian tubes in order to better understand the distribution of normalized discriminant function scores for the nuclei of fallopian tube epithelium representing women at normal risk cases (in grey) and women at high risk cases (in black). The discriminant analysis utilized only karyometric features that maximally reduced Wilks’ lambda (i.e., maximally separated high-risk from normal-risk epithelium). There is a significant separation of the two curves ($p<0.05$). A shift to the lower score values as seen in the black bars indicates higher deviation from normal.
stand the difference between high-risk and normal-risk nuclear features.

Finally, we are collaborating with biomedical engineers at the University of Arizona in the development of a novel miniature endoscopic device that can be threaded through the vagina and into the fallopian tubes to collect fallopian tube cells. This revolutionary device would eliminate the need to surgically remove the tubes in order to accurately assess cancer risk. With the assistance of this device, we are hopeful that karyometry can offer a novel and noninvasive mechanism for ovarian cancer screening, thereby encouraging noninvasive prevention strategies and early detection of the disease, ultimately contributing to precision oncology in gynecological malignancies.

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Contributions

Dr. Samantha Russell collated the karyometric data and composed the manuscript. Dr. Gustavo Rodriguez collected all the participant tissue samples, facilitated the production of the histopathological slides, and provided scientific input to the project and manuscript. Dr. Hao Helen Zhang provided statistical advising, data interpretation, and assistance throughout the editing process. Michael Yozwiak provided oversight on the segmentation of the microscopic studies and input into the interpretation of the generated data. Dr. Charmi Patel provided histopathological review of all samples and input into selection of microscopic images for karyometric analyses. Dr. Melody Maarouf provided the segmentation of all histopathological specimens. Hubert Bartels collected and maintained all analytical data. Dr. Jennifer Barton participated in the discussion, interpretation of study data, and editing of the manuscript as well as potential application to the future collection of tissue samples. Ahyoung Amy Kim provided statistical input throughout the editing process and participated in the discussion. Sri Saii Atluri participated in discussion of data and has been integral in continuing segmentation of fallopian tubes for future projects. Dr. Peter Bartels presided over the final statistical analyses and the interpretation of results. Dr. David Alberts developed experimental design, supervised all laboratory functions, and provided major input into the manuscript drafts.

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