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Effects of biographical variables on cervical fluorescence emission spectra

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Abstract. Diagnostic algorithms can classify tissue samples as diseased or nondiseased based on fluorescence emission collected from the intact cervix. Such algorithms can distinguish high-grade squamous intraepithelial lesions from low-grade squamous intraepithelial lesions. An understanding of the effects of the values of biographical covariates, such as age, race, smoking, or menopausal status on the emission spectra for each patient could improve diagnostic efficiency. The analysis described was performed using data collected from two previously published clinical trials; one study measured spectra from 395 sites in 95 patients referred to a colposcopy clinic with abnormal Pap smears, and the second study measured spectra from 204 sites in 54 patients self-referred for screening and expected to have a normal Pap smear. For this analysis, data about age, race, menstrual cycle, and smoking were collected. The principal components from normalized data were compared. There are clear intensity differences observed with age and menopausal status; postmenopausal patients exhibit higher emission intensities. Differences associated with biographical variables need to be tested in larger studies, which stratify adequately for these variables. The addition of these biographical variables in the preprocessing of data could dramatically improve algorithm performance and applicability. [DOI: 10.1117/1.1578642]

Keywords: clinical trial; algorithm; squamous intraepithelial lesions; fluorescence spectroscopy.

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1 Introduction

Diagnostic algorithms can classify tissue samples as diseased or nondiseased based on fluorescence emission collected from the intact cervix.[1–5] Such algorithms can discriminate normal tissue from squamous intraepithelial lesions (SILs) and low-grade SILs from high-grade SILs with a similar sensitivity and significantly improved specificity relative to colposcopy in expert hands.[6] However, it was noted in early clinical studies that there is great variability in the fluorescence spectra collected from different patients, even within a single histopathological category. For example, peak fluorescence intensities of normal tissues can vary by more than a factor of 5 from patient to patient, but within a single patient the standard deviation is usually less than 25% of the average value.

The potentially important chromophores in the cervix have been identified in the literature as the metabolic indicators reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD), the structural proteins collagen and elastin, and the oxyhemoglobin of blood. A model of cervical tissue autofluorescence shows that as disease progresses, increased contributions from NADH fluorescence and oxyhemoglobin absorption are observed, while a decreased contribution is seen from the structural proteins.[2]

It was recognized during early clinical studies that the measured fluorescence intensity in the cervix was often greater from older women.[7] While age-related structural changes in collagen are well documented in several tissues,[8–11] quantitative results of dermal collagen content with age are quite divergent—indicating an increase, decrease, or no change with age.[8–11] Moragas et al.[8] showed that dermal thickness and the spatial density of dermal collagen increase progressively until about age 40 and that there is a sharp decrease after age 50. Bailey et al.[9] associate the deleterious age-related changes in collagen to the intermolecular cross-linking of the collagen molecules within the tissues. Rechberger et al.[10] tested the hypothesis that female stress incontinence is due to weakening of the collagen framework of the pubocervical fascia and found that the fascia of women with incontinence contained 20% less collagen than normal. While incontinence is more common in older women, Rechberger's study found no significant change in collagen content with age or menopausal status.

There are also studies in the literature that describe structural differences in tissues among individuals of different races. Keloids are benign proliferative growths of dermal collagen that usually result from excessive collagen formation during dermal wound healing. African-Americans form keloids more often than Caucasians, with the reported ratios of

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incidence varying from 2:1 reported by Brenizer to 19:1 reported by Fox.[12–14] Arnold and Grauer found that in Hawaii, keloids are five times more likely in Japanese individuals and three times more common in Chinese individuals than among Caucasians.[12] These racial differences are not fully understood, but there appears to be a relationship between keloids and abnormal melanocyte-stimulation hormone. Meyer et al.[11] described racial differences in the connective tissue components of the aorta and coronary arteries between white and nonwhite South Africans. After the age of 40, calcium concentration in the aorta increased much more rapidly in whites than in Bantu. The tendency for collagen content to decrease with age was more pronounced in the aorta and coronary arteries of white males than in corresponding Bantu males.[11]

In addition to the racial and age-related differences that may be expected based on the studies in other tissue types, fluorescence signals may be affected by the hormonal fluctuations that occur during a normal menstrual cycle and particularly during pregnancy and menopause. In postmenopausal women, the cervical epithelium may become thinner and appear atrophic.[15]

Smoking is a known risk factor for various types of cancer, possibly including cervical cancer, and may cause early morphological changes in tissue that still appears normal. The goal of this study was to explore possible correlations between fluorescence emission spectra and biographical characteristics of the patients, including age, race, menopausal status, and smoking.

2 Methods

The analysis described here was performed using data collected in two previously published studies.[5,16] Ramanujam et al.[5] measured cervical fluorescence *in vivo* at 337, 380, and 460 nm excitation from 395 sites in 95 patients with a history of SIL (study 1). Biopsies were taken from the abnormal-appearing areas and processed for standard pathologic evaluation. For these patients, data include age, race, stage in menstrual cycle, hormonal status, method of birth control, gravidity, human papilloma virus (HPV) status, and smoking status were also collected. Brookner et al.[16] collected fluorescence emissions at the same three excitation wavelengths from 204 sites in 54 female volunteers with no current gynecologic symptoms and no history of dysplasia (study 2). Pap smears were taken from these women, and any participants with abnormal test results were referred for further evaluation. For each participant, the age, race, stage in menstrual cycle, hormonal status, method of birth control, gravidity, and smoking status were noted.

The spectroscopic system used to measure the fluorescence spectra *in vivo* has previously been described in detail.[3] Briefly, this device consists of two dye lasers pumped by a nitrogen laser, an optical fiber probe, and an optical multichannel analyzer. Minor system modifications were made prior to the study involving normal volunteers, and these changes have also been described previously.[16] Fluorescence intensity was measured from a standard rhodamine solution prior to measurement of each patient for calibration purposes.[3,16] Correction factors were obtained for both versions of the collection system, and the data from each study were multiplied by the appropriate correction factors prior to the analysis described here.

3 Data Analysis

The data from each study were multiplied by the appropriate correction factors and divided by the peak intensity of the rhodamine standard. From each of the studies described, the measurements taken from sites classified as squamous normal (SN) or squamous intraepithelial lesions were used for the statistical analysis. The SIL measurements were all obtained from biopsy-proven SIL areas in women seen in the referral setting. The following biographical covariates were included in the analysis: race (white versus other), age (less than 40 versus greater than or equal to 40), current smoker (yes or no), menopausal status (pre versus post).

The data were divided into three sets for analysis: SN sites from the screening study, SN sites from the referral study, and SIL sites from the referral study. A given patient can thus occur in both of the last two studies. The SN and SIL data were separated so that we conclude focus on the effects of the biological covariates and avoid possible complications caused by interactions with disease status. The SN sites from the two studies were not combined since even SN tissue in women with cervical abnormalities may show preclinical changes.

The data for each emission spectrum for each excitation wavelength were normalized to have a maximum value of 1.0. The maximum values of the unnormalized data were also retained for analysis, as were the emission frequencies at which the maximum occurred. The emission spectra for each excitation wavelength were then concatenated into data matrices; the rows of the matrices correspond to the spectra from individual sites and the columns correspond to specific emission wavelengths. The data from all three datasets were included in each matrix. There were therefore three data matrices, corresponding to the three excitation wavelengths.

The principal components were computed for each data matrix; the scores associated with the components necessary to account for 99% of the variability of the data were retained for analysis: six components were retained for excitation wavelength 337 nm, ten for excitation wavelength 380 nm, and eleven for excitation wavelength 460 nm. Since the maximum emission intensity and the position of the maximum were also retained for each of the three excitation wavelengths, there are a total of $6+10+11+6=33$ outcomes to compare for each dataset. Although the datasets are analyzed separately, as explained earlier, the principal components were computed for the combined data so that the results from each dataset could be meaningfully compared.

For each outcome variable, comparisons were based on the biographical covariates. The statistic used for comparison was the Z score computed from the Wilcoxon rank-sum statistic. The Z score was used rather than the raw rank sum since there were missing values for some covariates; the Z score does not reflect as strongly the number of cases used in the comparison. The probability values for the rank-sum statistics were based on comparison with 10,000 randomly generated permutations of the data. All sites for a given patient were permuted as a unit, thus preserving the matching inherent in the data. Multiplicity corrections for the large number of P values com-

Table 1 Number of cases in each dataset by value of biographical covariates.

Screening study – SN				
	Current smoker	Age > 40	White	Postmenopausal
No	44	31	11	40
Yes	6	19	24	7
Total	50	50	35	47
Referral study – SN				
	Current smoker	Age > 40	White	Postmenopausal
No	47	79	40	75
Yes	44	12	51	3
Total	91	91	91	78
Referral study – SIL				
	Current smoker	Age > 40	White	Postmenopausal
No	31	58	29	49
Yes	33	6	35	2
Total	64	64	64	51

puted used the method of Westfall and Young;[17] the multiplicity correction was performed separately for each dataset.

4 Results

Table 1 shows the number of patients included in the analyses for each dataset, broken down by the values of the biographical covariates. Since there were so few postmenopausal patients available in the referral datasets, this variable was not included in the analysis of those datasets. Table 2 shows the number of individual sites included in each dataset, broken down by the values of the biographical covariates.

Table 2 Number of sites in each dataset by value of biographical covariates.

Screening study – SN				
	Current smoker	Age > 40	White	Postmenopausal
No	93	67	22	86
Yes	13	39	52	14
Total	106	106	74	100
Referral study – SN				
	Current smoker	Age > 40	White	Postmenopausal
No	98	163	83	153
Yes	89	24	104	7
Total	187	187	187	160
Referral study – SIL				
	Current smoker	Age > 40	White	Postmenopausal
No	55	110	48	97
Yes	64	9	71	4
Total	119	119	119	101

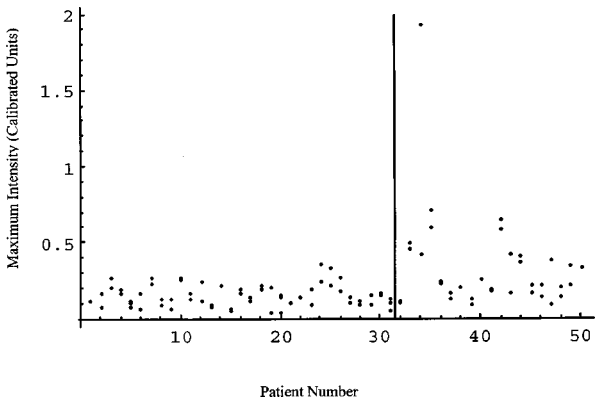

Fig. 1 Maximum emission intensity for an excitation wavelength of 460 nm. Patients to the left of the vertical line are less than 40 years old; patients to the right are more than 40 years old. The x-axis gives the patient identifier; points lying on a vertical line come from repeated measures of the same patient. The values from patients less than 40 years differ significantly from the values for patients greater than 40 ($P=0.048$).

After adjustment for multiplicity, both menopausal status and age showed significant effects at the 5% level on the outcome variables in the screening SN dataset. Specifically, age shows an effect on the maximum emission intensity for an excitation wavelength of 460 nm ($P=0.048$), and menopausal status shows a significant effect on the maximum emission intensity for an excitation of 460 nm ($P=0.012$) and on the scores for principal components 2 and 8 for an excitation wavelength of 380 nm ($P=0.012$ and $P=0.030$). Since the eighth principal component accounts for only 0.6% of the variability of the data, any change in its value is unlikely to be useful in discrimination; however, the second principal component accounts for 22.4% of the variability, and differences in its score can have substantial effects. For the SN data from the referral study, none of the covariates showed any significant effect for any of the outcomes. For the SIL data from the

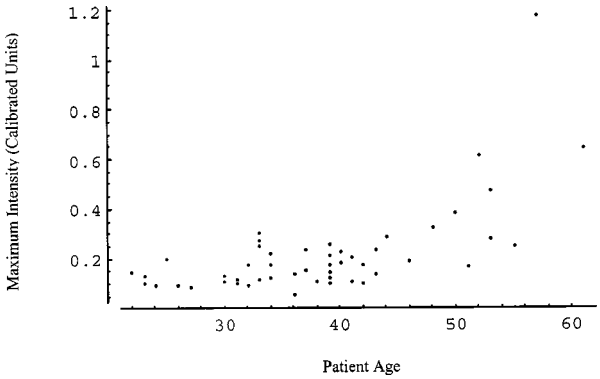

Fig. 2 Mean maximum emission intensity for an excitation wavelength of 460 nm per patient versus age.

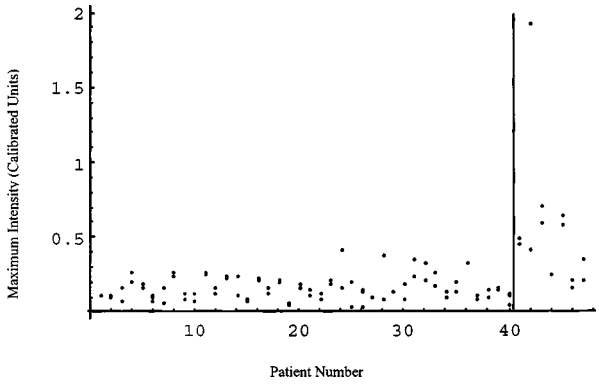

Fig. 3 Maximum emission intensity for an excitation wavelength of 460 nm. Patients to the left of the vertical line are premenopausal; patients to the right are postmenopausal. The *x*-axis gives the patient identifier; points lying on a vertical line come from repeated measures of the same patient. The values from premenopausal patients differ significantly from the values for postmenopausal patients ($P = 0.012$).

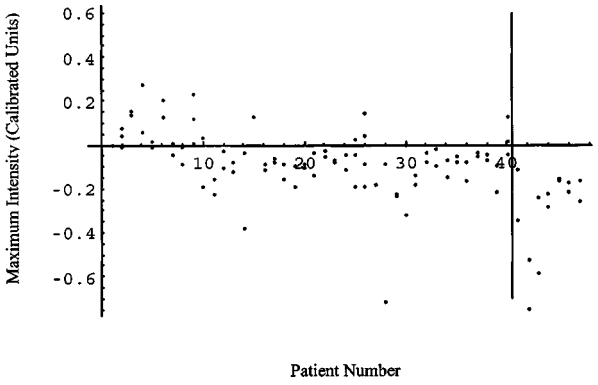

Fig. 5 Score for principal component 2 of an excitation wavelength of 380 nm. Patients to the left of the vertical line are premenopausal; patients to the right are postmenopausal. The *x*-axis gives the patient identifier; points lying on a vertical line come from repeated measures of the same patient. The values from premenopausal patients differ significantly from the values for postmenopausal patients ($P = 0.012$).

referral study, smoking showed a significant effect on the scores for the sixth principal component from excitation 380 nm ($P = 0.052$); again, this component accounts for only a very small proportion (0.9%) of the variability. The lack of significant effects for age and menopausal status in the referral datasets may be due to the small number of older postmenopausal women contained in those data sets.

Figures 1–6 illustrate the effects of the covariates found to be significant in the screening SN dataset; principal component 8 is not shown because of the small proportion of variability attributable to it. Figure 1 demonstrates the effect of age on the maximum emission intensity for an excitation wavelength of 460 nm. The data for each sampled site are plotted; the patient sequence number is given on the *x*-axis so that points from the same patient lie on the same vertical line. The points to the left of the vertical line marked on the graph are from patients less than 40 years old. Figure 2 plots the average value of maximum emission intensity for excitation wavelength of 460 nm for each case versus age. The trend toward increasing intensities with increasing age is clear.

Figure 3 shows the effect of menopausal status on maximum emission intensity for an excitation wavelength of 460 nm. Points to the left of the vertical line are from premenopausal patients. Figure 4 is similar, but the average intensity is plotted for each patient. This both clarifies the trend to increased intensity after menopause and illustrates the within-patient variability of the data.

Figure 5 shows the effect of menopausal status on the score associated with the second principal component of the emission spectra from an excitation wavelength of 380 nm. Figure 6 is similar, but with the mean value plotted for each patient. The scores are noticeably lower for the postmenopausal patients.

5 Discussion and Conclusions

Fluorescence spectroscopy may be implemented in the future to aid the detection and diagnosis of early-stage disease in many organ sites. This technique is advantageous because it may allow tissue classification in near-real time without the removal of tissue. However, the interaction of light and tissue is extremely complex and only partially understood. Changes that occur in tissue biochemistry and morphology during the

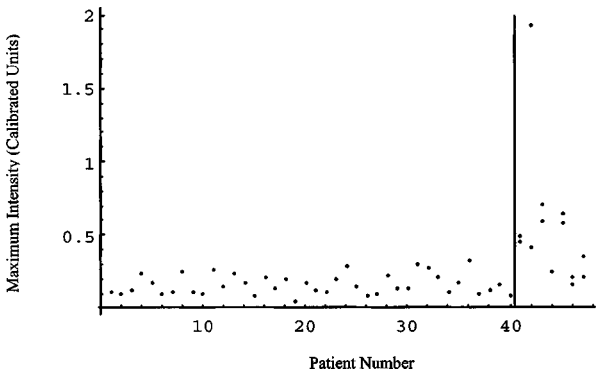

Fig. 4 Mean maximum emission intensity for an excitation wavelength of 460 nm per patient. Patients to the left of the vertical line are premenopausal; patients to the right are postmenopausal. The *x*-axis gives the patient identifier; there is one point per patient.

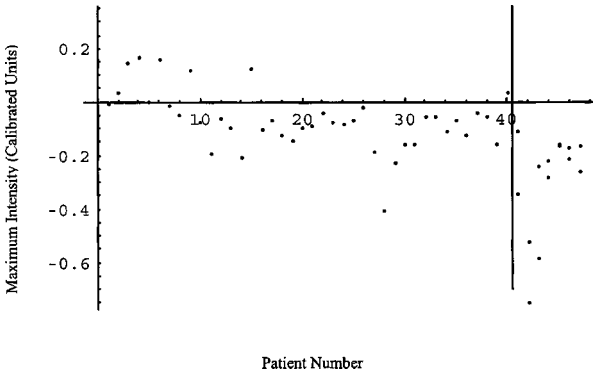

Fig. 6 Mean score for principal component 2 of an excitation wavelength of 380 nm per case. Patients to the left of the vertical line are premenopausal; patients to the right are postmenopausal. The *x*-axis gives the patient identifier; there is one point per patient.

progression of disease can result in changes in the fluorescence spectra measured at the tissue surface, and such differences can be exploited to classify the tissue. However, tissue biochemistry and morphology can differ among patients for reasons other than the presence or absence of disease. For example, the cervical epithelium undergoes changes in response to different hormone levels, particularly during pregnancy and menopause. Age and race-related differences have been reported in the collagen content of the dermis and other tissues.[8–12] Previously developed algorithms that classify tissue based on fluorescence spectra have not, to our knowledge, considered any patient variable other than the pathological diagnosis of the site that was spectroscopically interrogated.

The results described in this study demonstrate that statistically significant differences in the principal components that describe spectral data can arise from factors such as the patient's age or menopausal status, even when the pathological diagnosis of the measured tissue is the same. The increased fluorescence intensity with increasing age and menopausal status is consistent with a large proportion of the signal originating in the stroma, which contains collagen and elastin. Older, postmenopausal women are known to experience a thinning of the cervical epithelium, and may experience changes in collagen cross-linking as well.[9,15] Further statistical work using a larger dataset is necessary to gain a complete understanding of the effects of biographical variables on fluorescence spectra. In particular, a greater number of measurements are needed from older, postmenopausal women. Future clinical trials are planned in which the participants will be stratified by their age and race to ensure that the data are from a group that is well distributed across these variables. This will permit development and training of an algorithm that can account for such interpatient variations. However, the differences in spectral data presented here may be utilized to develop new, more rational preprocessing techniques. Current preprocessing techniques, normalization and mean scaling, are limiting because normalization ignores intensity differences between spectra and mean scaling requires an equal number of normal and abnormal sites per patient. Alternative preprocessing methods, which could account for the differences that are due to patient age or menopausal status without normalization, for example, should offer dramatic improvements to algorithm performance and applicability.

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