

OPEN

Label-Free Imaging of Female Genital Tract Melanocytic Lesions With Pump-Probe Microscopy: A Promising Diagnostic Tool

Francisco E. Robles, PhD,^{1,2} Sanghamitra Deb, PhD,¹ Martin C. Fischer, PhD,¹
Warren S. Warren, PhD,¹ and Maria Angelica Selim, MD³

Objectives: Melanomas of the female genital tract present a unique clinical challenge. Not only are these lesions in an anatomically sensitive area, but also they tend to be multifocal and have high recurrence rates. Furthermore, several benign melanocytic proliferations resemble early-stage melanoma clinically and/or histopathologically. Thus, there is a significant need for additional tools that can help correctly diagnose and stage these lesions. Here, we quantitatively and nondestructively analyze the chemical composition of melanin in excised pigmented lesions of the female genital tract using pump-probe microscopy, a high-resolution optical imaging technique that is sensitive to many biochemical properties of melanin.

Materials and Methods: Thirty-one thin (~5 μm) tissue sections previously excised from female genital tract melanocytic lesions were imaged with pump-probe microscopy and analyzed.

Results: We find significant quantitative differences in melanin type and structure between melanoma and nonmalignant melanocytic proliferations. Our analysis also suggests a link between the molecular signatures of melanins and lesion-specific genetic mutations. Finally, significant differences are found between metastatic and nonmetastatic melanomas. The limitations of this work include the fact that molecular information is restricted to melanin pigment and the sample size is relatively small.

Conclusions: Pump-probe microscopy provides unique information regarding the biochemical composition of genital tract melanocytic lesions, which can be used to improve the diagnosis and staging of vulvar melanomas.

Key Words: vulvar melanoma, histopathology, prognosis, quantitative molecular imaging, nonlinear microscopy

(*J Low Genit Tract Dis* 2017;21: 137–144)

Malignant melanoma of the vulva accounts for 2% to 7% of all melanomas in women^{1,2} and is the second most common malignancy afflicting the region.³ Similar to other cutaneous

melanomas, melanomas of genital mucosal tissue originate from melanocytes in the basal layer; however, various studies have found important biological differences. For example, vulvar melanoma is a multifocal disease, often subclinical, with a high recurrence rate (30%–50%) that is associated not with inadequate surgical control but rather from not-well-understood disorders of the melanocytes in the genital squamous epithelium.¹ Vulvar melanomas are also highly metastatic and have a worse prognosis than other cutaneous melanomas, with an estimated 5-year survival rate of 40% to 60%.^{3,4} Furthermore, differentiating melanoma from benign lesions in the vulva is critical because of the difficulty involved in performing local excisions and the risk of complications that can lead to sexual or urinary dysfunction. For these reasons, vulvar melanomas require different considerations for diagnosis and treatment compared with other melanomas.

Identification of this disease can be complicated by a number of benign melanocytic proliferations, including melanotic macules and atypical genital nevi (AGN). Melanotic macules are highly pigmented macules, typically large (>5 cm), with irregular borders that commonly develop in multiples.⁵ These lesions can be clinically indistinguishable from melanomas, and a biopsy must be performed to confirm their diagnosis.⁵ This problem is exacerbated by the fact that melanotic macules are extremely common; they are estimated to develop in one of ten women.^{5–7} Atypical genital nevi are less common but can be difficult to distinguish from melanoma both clinically and histopathologically (using hematoxylin-eosin [H&E] stains), because the two types of lesions can have overlapping histologic features.^{5,8,9} Given (1) the sensitivity of this anatomical site, (2) the high recurrence rate as well as multifocal and subclinical nature of vulvar melanomas, and (3) the relatively high incidence rate of benign pigmented lesions that resemble early-stage malignant melanoma, there is a significant need for additional tools that can help correctly diagnose these lesions.

Label-free optical imaging methods, which do not use exogenous labels for contrast, with molecular sensitivity and high spatial resolution have emerged as powerful tools for identifying disease because they can probe important endogenous biochemical properties without destroying the samples or disrupting the biological environment. Here, we apply pump-probe microscopy, an emerging nonlinear optical technique, to quantitatively image the biochemical composition of melanin in melanocytic female genital tract lesions. The rich biochemical information provided by this method can be used as an indicator of melanocyte activity, which in turn reflects the status of melanocytic lesions.

Pump-probe microscopy interrogates the ultrafast transient excited- and ground-state photodynamics of pigmented molecules with subcellular spatial resolution.^{10,11} Although the linear absorption properties of different melanins (eumelanin and pheomelanin) are featureless and similar—if not identical—to one another, the ultrafast transient properties of these large, heterogeneous biopolymers exhibit remarkable differences; previously,

¹Department of Chemistry, Duke University, Durham, NC; ²Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory School of Medicine, Atlanta, GA; and ³Department of Pathology, Duke University Medical Center, Durham, NC

Correspondence to: Warren S. Warren, PhD, Department of Chemistry, Duke University, French Family Science Center, Box 90346, Durham, NC 27708.
E-mail: warren.warren@duke.edu

The authors have declared they have no conflicts of interest.

The study was supported by NIH R01-CA166555 (M.A.S., W.S.W.), NSF CHE-1309017 (M.C.F.), the Burroughs Wellcome Fund 1012639 (F.E.R.), NIH F32CA183204 (F.E.R.), the Duke Center for In vivo Microscopy NIH P41EB015897, and Duke University.

This work is under an institutional review board exempt protocol (exemption 4). Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.jlgt.com).

Copyright © 2017 The Author(s). Published by Wolters Kluwer Health, Inc. on behalf of the American Society for Colposcopy and Cervical Pathology. This is an open-access article distributed under the terms of the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CCBY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.

DOI: 10.1097/LGT.0000000000000290

we have shown that the transient response depends on melanin type, oxidation state, aggregate size, and metal content.¹² This rich source of molecular contrast, with high spatial resolution, is uniquely available with pump-probe microscopy^{10,13} and is ideal for studying melanocytic lesions. This method has shown significant promise in differentiating melanomas from cutaneous melanocytic nevi^{10,14} and conjunctival melanoma from primary acquired melanosis,¹⁵ as well as providing novel insight into the metastatic potential of invasive cutaneous melanomas.¹⁶

This study presents a quantitative analysis of the biochemical composition and structure of melanin in thin, unstained tissue sections of female genital tract melanocytic lesions. Data show significant differences between melanomas and benign melanocytic proliferations, as well as metastatic and nonmetastatic melanomas. We also draw a correlation between molecular signatures and lesion-specific genetic mutations. These results can help improve diagnosis and staging. Finally, we discuss the feasibility for identifying these lesions in vivo.

METHODS

In this study, we analyze thirty-one previously excised female genital tract melanocytic lesions (Table 1). From each fixed tissue specimen, two adjacent, thin (~5 μm) sections were prepared: one unstained and one stained with H&E. A board-certified dermatopathologist used the H&E-stained sections to classify regions of interest (no discrepancies were found in the samples' evaluation compared with the original diagnosis found in the medical records). These regions were identified in the adjacent, unstained sections and imaged with pump-probe microscopy (note that neither the fixation nor storing of the samples alters the melanin pump-probe signals—see Supplemental Digital Content (<http://links.lww.com/LGT/A61>) for more details) A total of 612 pump-probe images were acquired.

The pump-probe system (see Figure S1) has been described in detail elsewhere.^{10,11,16} In short, we integrated a commercial laser-scanning microscope (Zeiss, LSM 510; using a 20 \times [0.8NA] objective) with a custom femtosecond laser setup that yields access to the femtosecond to picosecond transient dynamics. The laser pulses use less than 0.65 mW total power to avoid any effects that may alter the melanin pigment chemistry (e.g., photobleaching).¹² The data acquired are three-dimensional, with two dimensions being the x and y coordinates of the thin sample,

TABLE 1. Sample Set Characteristics

	No. images*	Sections with tissue type	Patient diagnosis
Normal	30	1	0
Melanotic macule	103	11	11
Atypical genital nevi	91	7	7
AMH	54	6	1
Melanoma in situ	219	7	2
Invasive melanoma	115	7	10
Total	612	39	31

*Each image region is a 420 \times 420 μm^2 area.

The table should be read as follows: using invasive melanomas as an example, the sample set contained 10 specimens from patients diagnosed with invasive melanoma, but only seven of the available/analyzed sections contained any indication of invasive disease. The other three only showed features consistent with melanoma in situ. A total of 115 images were taken from regions with invasive melanoma.

AMH indicates atypical melanocytic hyperplasia.

and the third being the ultrafast transient dynamics (i.e., molecular signatures). This is illustrated in Figure 1F. Each individual pump-probe image has a field of view of 420 \times 420 μm (see Figure S1, Supplemental Digital Content, <http://links.lww.com/LGT/A61>).

The transient dynamics (i.e., molecular signatures) are quantified using a geometrical representation of principal component (PC) analysis.¹⁶ Here, the space spanned by the data's top three PCs (see Figure 1G), which account for 98% of the variance, is represented in spherical coordinates (inset of Figure 1G). In this coordinate system, the angles (θ and ϕ) describe the transient dynamics, whereas the radius (R) represents the signal strength (i.e., relative concentration). In these data, two endmembers are observed: one is located at an azimuth angle of $\theta = 0$ rad (i.e., a positive contribution from PC1), which signifies an excited state absorption interaction. We have observed these types of signals from melanins with large aggregate size and melanins void of metals. The other endmember lies around $\theta = 2.5$ rad (negative contribution from PC1, plus a positive contribution from PC2) and indicates a ground-state bleaching interaction. These signals have been observed in melanins with high levels of metal content, small aggregate size, and synthetic pheomelanin. All signals are effectively described through a linear combination of these two endmembers.

For image display, we use a hue-saturation-value color map scheme. The hue is determined based on θ (see Figure 1H), the value is set by R , and the saturation is set to 1. The pump-probe images are superposed on top of confocal reflectance images to enable visualization of nonmelanocytic structures.

RESULTS

Pump-Probe Microscopy Images of Female Genital Tract Melanocytic Lesions

Figure 1 shows a representative sample containing regions indicative of atypical melanocytic hyperplasia (AMH), fully evolved melanoma, and normal pigmented tissue. The criteria used to identify melanoma in situ include (1) increased melanocytic density, (2) confluence, (3) severe atypia, and (4) pagetoid spread. Although there is no clear definition for AMH, we considered regions adjacent to a fully evolved melanoma that contained one or two of the previous criteria as AMH. The corresponding pump-probe images (from the adjacent unstained tissue section) exhibit clear differences between these regions. The AMH region (highlighted in yellow, see Figure 1) contains elevated pigmentation in the epidermis, particularly in the basal layer. Furthermore, the bottom of the rete ridges shows a distinct change in pigment chemistry, which is likely an indication of the melanocytes' progression toward melanoma cells. The melanoma region (highlighted in red, see Figure 1) shows a severe disruption in the pigment architecture, with heavy pigmentation that is highly heterogeneous in chemical composition and that extends deep into the dermis. Dermal melanocytic nests are present, and a significant inflammatory response is observed. Note that melanophages tend to possess melanin with strong long-lived excited state absorption signals¹³ (demarcated in red hues). Finally, this sample shows a clear delineation of the tumor margin (see Figure 1E). Note that the melanoma and normal regions are both highly pigmented, which may appear similar under clinical evaluation, but pump-probe microscopy reveals drastic differences in their biochemical composition, which underscores the method's diagnostic potential.

Figure 2 shows an AGN. This lesion exhibits dyscohesive nests of various sizes and lentiginous proliferations of single cells (identified with H&E). The pigmentation is elevated with several

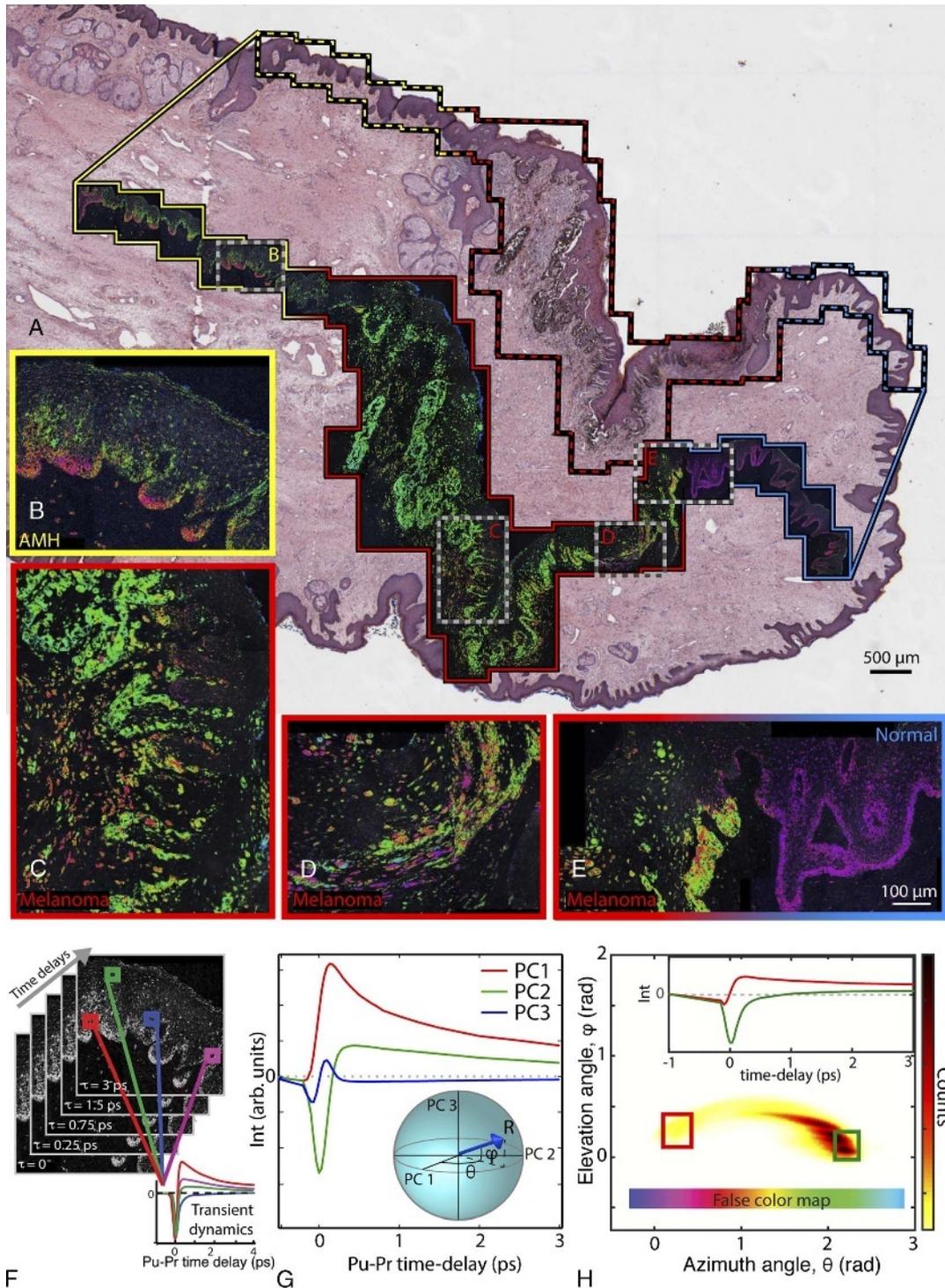


FIGURE 1. Vulvar melanoma. A–E, Pump-probe images from an *unstained* tissue section superposed with a photomicrograph of the adjacent H&E-stained tissue section. F, Schematic representation of a pump-probe image data cube. G, Principal components used to analyze the data. H, Two-dimensional histogram of the PCs' angular distribution. The color map at the bottom of the figure illustrates the false coloring scheme used for the pump-probe images. See text for details.

different types of spatial structures (i.e., heterogeneous); for instance, some areas contain melanin that appears large/coarse particularly in and around the nests, whereas other areas are more diffused and granular. The biochemistry composition, on the other hand, appears very homogeneous, characterized by a ground-state

bleaching signal (demarcated in green). This behavior is consistent across all AGN samples analyzed.

Figure 3 shows a representative melanotic macule. Here, we observe very organized structures with most of the pigment constrained to the dermal-epidermal junction. There is some

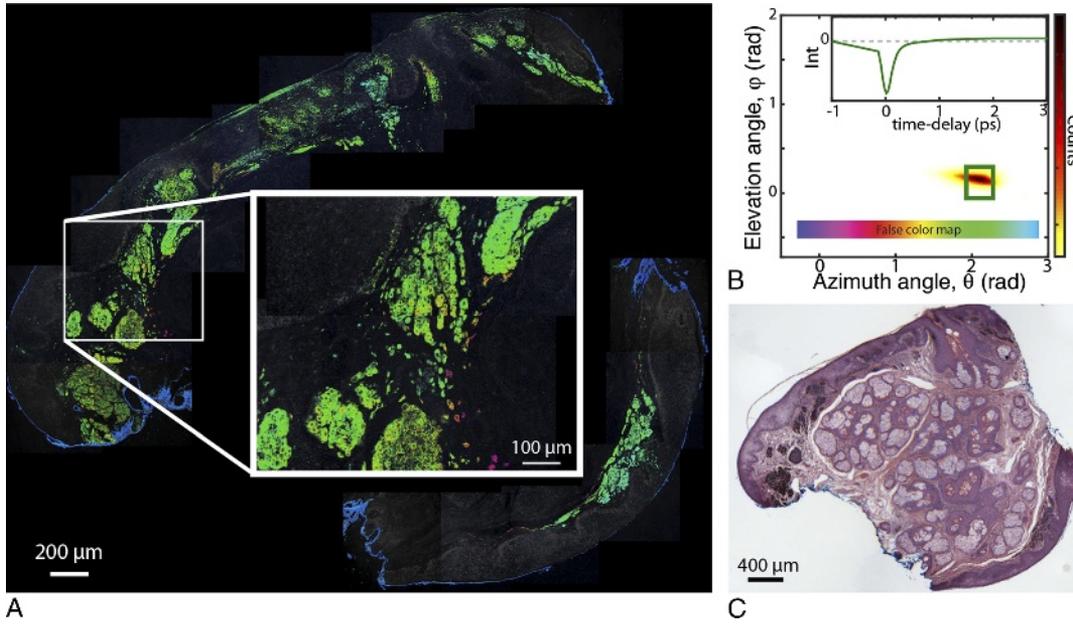


FIGURE 2. Atypical genital nevus. A, Pump-probe images and (C) photomicrograph of H&E-stained tissue sections of a representative atypical genital nevus. B, Two-dimensional histogram of the PCs' angular distribution.

melanin incontinence, but no melanocytic nests are present. Bridging of the rete ridges is observed, but the pigment is not extensive outside of the basal layer. These types of structures were found to be consistent across all melanotic macules

investigated but were not unique to this type of lesion. Finally, the low chemical heterogeneity found in this particular case is not a general feature, and higher interlesion and intralesion biochemical variability was observed on other melanotic macules.

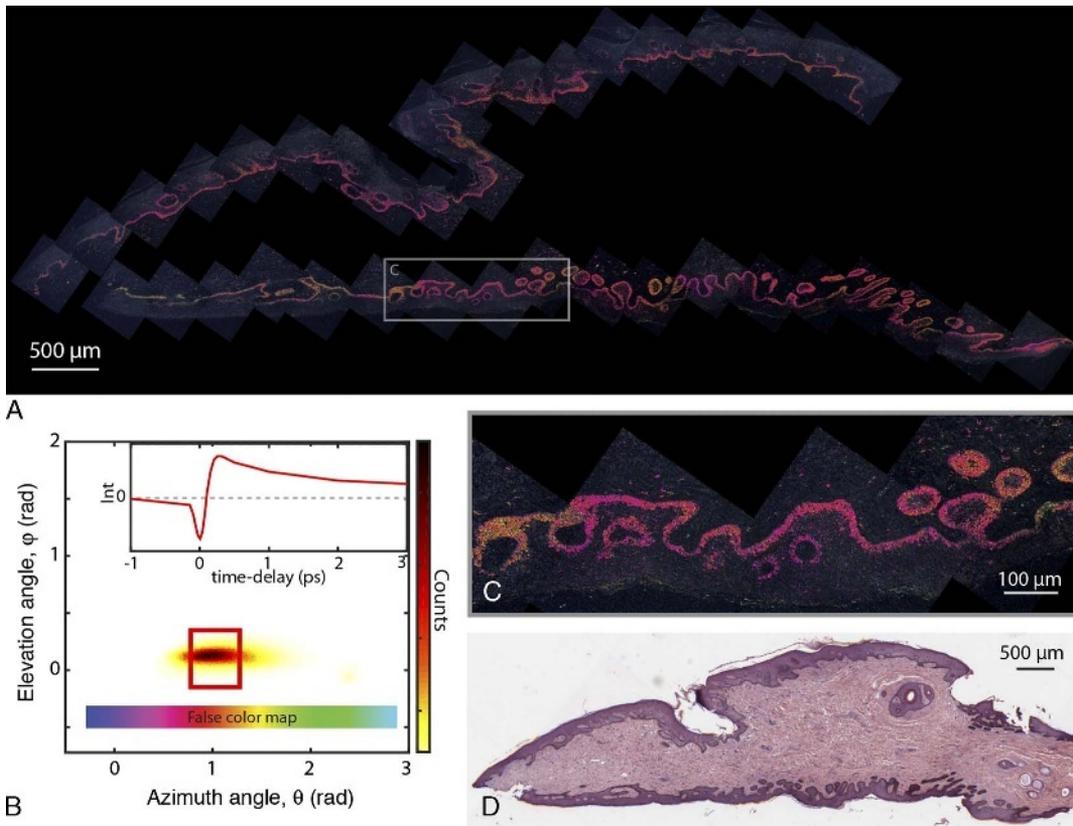


FIGURE 3. Melanotic macule. A and C, Pump-probe images and (D) photomicrograph of H&E-stained tissue sections of a representative melanotic macule. B, Two-dimensional histogram of the PCs' angular distribution.

Image Quantification and Multivariate Analysis

Melanin structure is quantified using principles of mathematical morphology.^{14,16,17} Briefly, the pump-probe data are used to render three gray scale images. One image is weighted by the pump-probe signal strength, R, which captures the overall structure of the melanin pigment (i.e., chemical composition is suppressed). The other two gray scale images are weighted by the relative concentration of the two endmembers. Specifically, the R image is multiplied by 1 for pixels with $\theta = 2.5$ and 0 for pixels with $\theta = 0$ rad for one image (denoted $\theta+$), and the other uses the opposite scale (denoted $\theta-$). These two images capture both the structural and biochemical information of the melanin. Finally, we apply a two-dimensional mathematical autocorrelation transformation to quantify the structure (See Supplemental Digital Content, <http://links.lww.com/LGT/A61>).¹⁴

Thirty different features were extracted to parameterize the image structure alongside the biochemical composition. A feature selection algorithm was then used to identify a subset of features

that minimized the misclassification error between melanomas and benign melanocytic proliferations. Seven features were selected, summarized in Figure 4, indicating that both the chemical composition and the subcellular structures are important for identifying disease. There are several interesting trends of the selected parameters: first, the mean, standard deviation, and entropy of the azimuth angle (all average measures of the pump-probe response) show that all lesion types have high biochemical heterogeneity with the unique exception of AGN. Next, the anisotropy of the $-\theta$ maps, which provide a measure of the spatial organization of pigments with low θ value (melanins with large aggregate size and/or void of metals), shows a trend of decreasing organization from normal tissue to melanoma, suggesting that the structure of this type of pigment degrades with increasing malignancy. Again, AGN are the exception, which shows higher variability in their spatial structure.

To estimate the predictive power, we use the leave-one-out cross-validation (LOOCV) method with individual lesions (i.e., all images from a patient) used as the test set and all others as

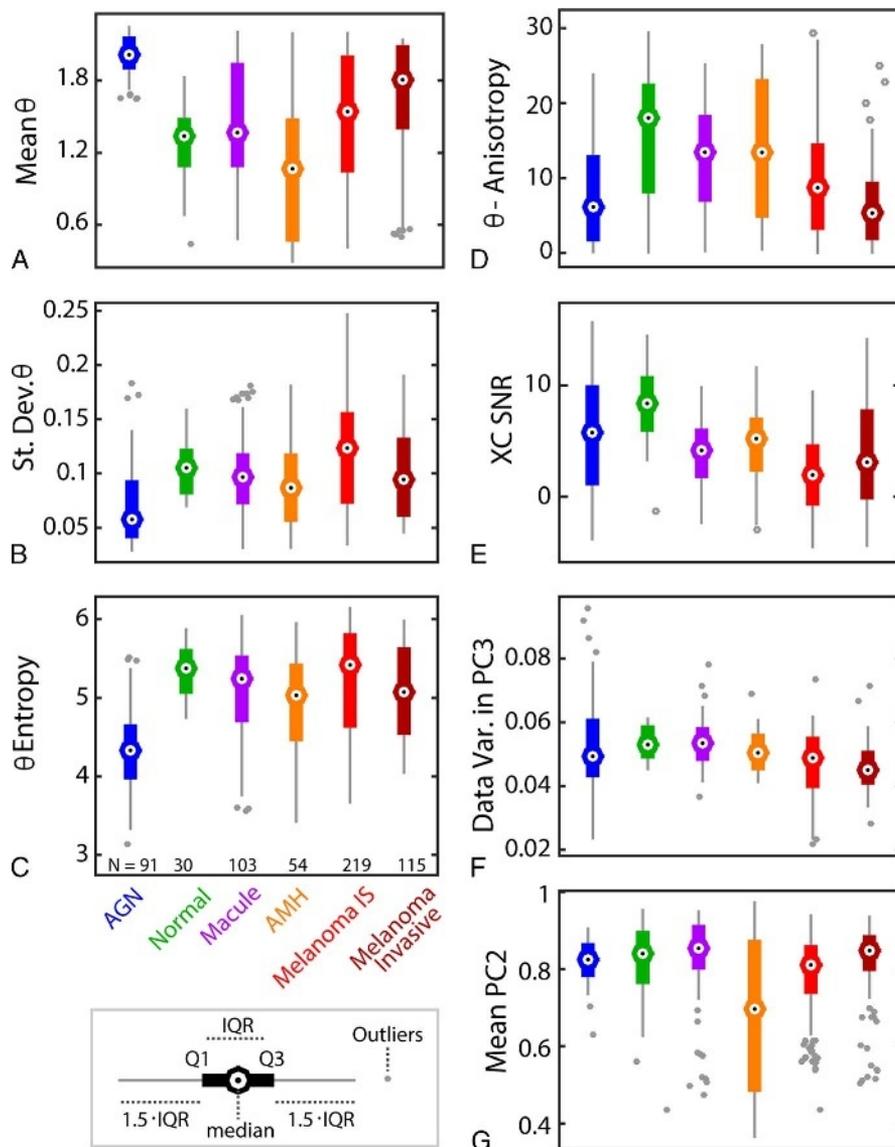


FIGURE 4. A–G, Summary of selected features used to differentiate between melanomas and benign melanocytic proliferations. IQR indicates inner quartile; Q, quartile; SNR, signal-to-noise ratio; var., variance; XC, cross correlation.

TABLE 2. Sensitivity and Specificity as Determined by LOOCV

	Optimized parameters, %		Structural parameters, %		No. lesions
	SE	SP	SE	SP	
Macule vs melanoma	78	70	72	71	23
AGN vs melanoma	90	70	70	43	19
AMH vs melanoma	89	76	73	39	13
Nonmelanoma vs melanoma	80	75	63	63	31
Nonmetastatic vs metastatic melanoma	76	82	50	39	8

SE indicates sensitivity; SP, specificity; AGN, atypical genital nevi; AMH, atypical melanocytic hyperplasia.

the training set. For this test, only two groups can be compared at a time (e.g., AGN vs melanoma, macules vs melanoma). The results (Table 2) show that the optimized parameters achieve an overall sensitivity of 80% and specificity of 75% for differentiating between benign melanocytic lesions and melanomas. The table also shows the results when using a different set of parameters that only quantify the pigment structure (see Figure 5). Here, the sensitivity and specificity for identifying melanoma versus nonmelanoma drops to 63%. The other statistical tests (AGN/AMH vs melanoma) also show drastic improvement in predictive power when including the biochemical information, except for melanotic macules versus melanoma where biochemical information seems less influential.

Finally, we compare metastatic melanomas (140 images from 4 lesions) versus nonmetastatic melanomas (74 images from 4 lesions). Using the same seven previous parameters, the LOOCV method yields a sensitivity and specificity of 70% and 66%, respectively. However, these parameters may not be optimal for differentiating between these two groups; thus, we repeat the feature selection algorithm. The selected features (see Figure 6) are primarily dependent on the biochemical composition of the melanin and show that metastatic melanomas have higher biochemical heterogeneity ($P < 0.001$ using the Bonferroni correction). Here, the second PC is primarily responsible for this behavior, suggesting that the ground-state bleaching signal is the most critical physical effect related to advanced disease. Repeating the LOOCV with the optimized features, we obtain a sensitivity and specificity toward metastatic disease of 76% and 82%, respectively (50% and 39%, without taking the biochemical information into accounting).

DISCUSSION

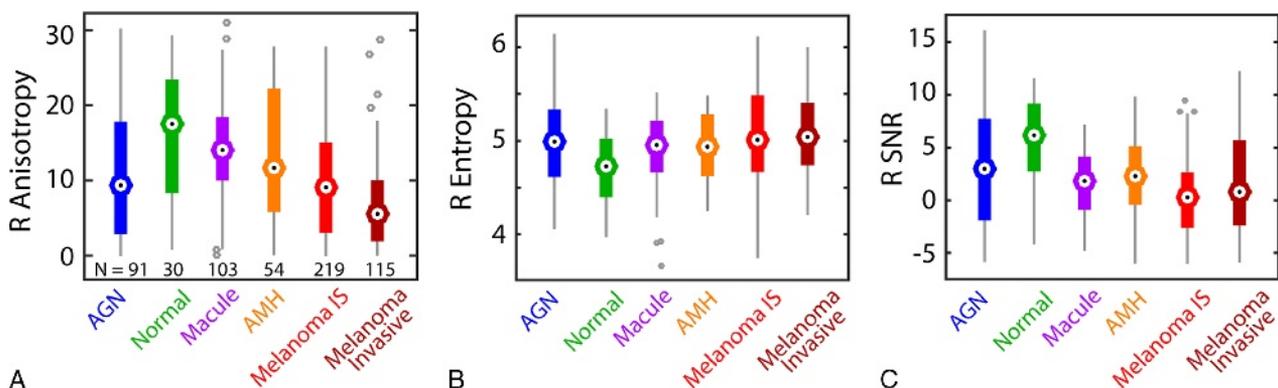
In this work, we have shown that the high-resolution, biochemical information provided by pump-probe microscopy can potentially help alleviate some of the challenges associated with diagnosing and staging vulvar melanomas. Although the focus of this study is on paraffin-embedded tissues, in vivo implementation is feasible¹⁸ and can in principle yield the same molecular information without the need for excisional biopsies (although the current technology has limited biochemical sensitivity in vivo¹⁹).

Our results provide unique insight into the most critical feature needed to evaluate female genital tract melanocytic lesions. Specifically, our data indicate that melanotic macules can be identified using the structure of the pigment alone, suggesting that current clinically available imaging technologies may be implemented to noninvasively identify these lesions. Indeed, preliminary results using reflectance confocal microscopy have shown some promise in evaluating melanotic macules.^{20,21} Nevertheless, the addition of the biochemical information improves the sensitivity from 72% to 78%. Thus, future implementation of pump-probe microscopy in vivo is likely to yield the most diagnostically rich information.

Unlike melanotic macules, the structure of AGN is highly heterogeneous and cannot be used alone to rule out melanoma. Initial studies using reflectance confocal microscopy have failed to improve clinical diagnosis of these lesions.²² On the other hand, our analysis shows that AGN exhibit a ubiquitous ground-state bleaching signal (potentially from melanins with high metal concentration, small aggregate size, and/or pheomelanin) that allows us to readily identify these lesions. Similarly, AMH could be differentiated from melanomas with high sensitivity and specificity using both structural and biochemical parameters.

The fact that 100% of the analyzed AGN possess the same molecular melanin signature suggests the first link between melanins' pump-probe response and a lesion's genetic mutations. Recent studies^{9,23} found that AGN very frequently contain BRAF V600F mutation (up to 100% of the time⁹), strongly suggesting that this mutation leads to pigment production predominantly characterized by ground-state bleaching. Note that BRAF mutations are rare in gynecologic melanomas, and if present, they are typically accompanied by other mutations,⁹ which likely alter the melanin biochemical composition and produce a less homogeneous pump-probe signal. Because no other type of lesion exhibits this behavior, it may be possible to not only identify AGN but also identify those rare occasions when they evolve to genital melanomas.

We found that the biochemical information can be used to evaluate metastatic potential with relatively high sensitivity and

**FIGURE 5.** A–C, Box plots of structural features (i.e., do not take the biochemical differences of melanin into account).

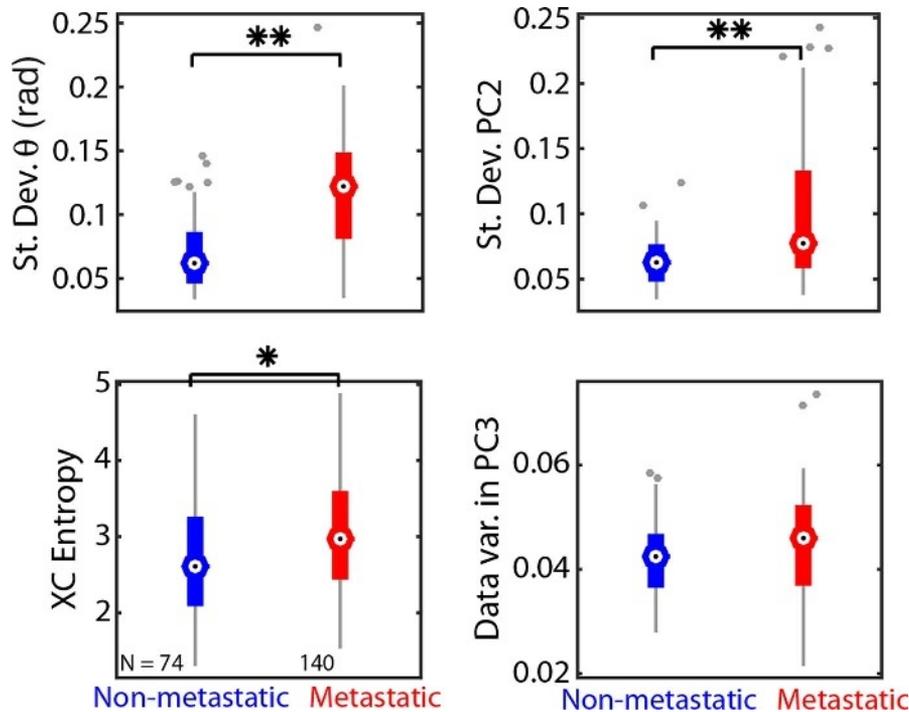


FIGURE 6. Selected features to differentiate between primary lesions of metastatic and nonmetastatic vulvar melanomas. * $p < 0.1$ and ** $p < 0.001$, using Bonferroni correction.

specificity. Even though the sample size was small, the results are significant because they further demonstrate the potential of pump-probe microscopy to stage melanomas on the basis of features of primary tumors,¹⁶ without resorting to more invasive procedures such as sentinel lymph node biopsies, the current clinical standard.

During the past few years, several noninvasive imaging devices have become available to facilitate the evaluation of pigmented lesions. Among these new technologies, pump-probe microscopy provides unique information regarding the biochemical composition of genital tract melanocytic lesions. Indeed, a larger, more comprehensive prospective study should be considered to more accurately assess the diagnostic utility of pump-probe microscopy; nevertheless, this study has shown the potential of this technology to improve the care of genital melanocytic lesions in the following ways: (1) as reflected by the molecular and clinical behavior, AGN can be readily differentiated from malignant melanomas; (2) large and numerous melanotic macules can potentially be evaluated without the need of multiple biopsies; (3) the border between melanoma and normal tissue can be delineated; and (4) metastatic potential can be evaluated from the primary tumor. Finally, with additional advances of compact, femtosecond laser sources, which would make it feasible to put a pump-probe system in a healthcare provider's office and a pathology laboratory, we expect that this noninvasive imaging technology can be incorporated in the daily clinical and pathological evaluation of pigmented lesions of the vulva leading to early diagnosis and avoidance of unnecessary procedures.

Role of Funding Sources

Our funding sources (NIH, NSF) provide support to explore, assess, and advance the capabilities of pump-probe microscopy, an imaging technology that can image specific cancer biomarkers in developing skin lesions. Our funding sources (NIH, BWF) also

support the career development activities for postdoctoral fellows in a degree-granting institution.

REFERENCES

1. Lotem M, Anteby S, Peretz T, et al. Mucosal melanoma of the female genital tract is a multifocal disorder. *Gynecol Oncol* 2003;88:45–50.
2. Trimble EL. Melanomas of the vulva and vagina. *Oncology (Williston Park)* 1996;10:1017–23.
3. Irvin WP Jr, Legallo RL, Stoler MH, et al. Vulvar melanoma: a retrospective analysis and literature review. *Gynecol Oncol* 2001;83:457–65.
4. Dunton CJ, Berd D. Vulvar melanoma, biologically different from other cutaneous melanomas. *Lancet* 1999;354:2013–4.
5. Massi G, LeBoit PE. *Histological Diagnosis of Nevi and Melanoma*. 2nd ed. New York: Springer; 2013.
6. Cengiz FP, Emiroglu N, Wellenhof RH. Dermoscopic and clinical features of pigmented skin lesions of the genital area. *An Bras Dermatol* 2015;90:178–83.
7. Hosler GA, Moresi JM, Barrett TL. Nevi with site-related atypia: a review of melanocytic nevi with atypical histologic features based on anatomic site. *J Cutan Pathol* 2008;35:889–98.
8. Yarak S. Clinical and histopathological characteristics of genital melanocytic nevi: a report of 109 cases and a review of the literature. *J Clin Exp Dermatol Res* 2013;3:1–6.
9. Tseng D, Kim J, Warrick A, et al. Oncogenic mutations in melanomas and benign melanocytic nevi of the female genital tract. *J Am Acad Dermatol* 2014;71:229–36.
10. Matthews TE, Piletic IR, Selim MA, et al. Pump-probe imaging differentiates melanoma from melanocytic nevi. *Sci Transl Med* 2011;3:71ra15.
11. Fu D, Matthews TE, Ye T, et al. Label-free in vivo optical imaging of microvasculature and oxygenation level. *J Biomed Opt* 2008;13:040503.

12. Simpson MJ, Wilson JW, Robles FE, et al. Near-infrared excited state dynamics of melanins: the effects of iron content, photo-damage, chemical oxidation, and aggregate size. *J Phys Chem A* 2014;118:993–1003.
13. Simpson MJ, Wilson JW, Phipps MA, et al. Nonlinear microscopy of eumelanin and pheomelanin with subcellular resolution. *J Invest Dermatol* 2013;133:1822–6.
14. Robles FE, Wilson JW, Warren WS. Quantifying melanin spatial distribution using pump-probe microscopy and a 2-D morphological autocorrelation transformation for melanoma diagnosis. *J Biomed Opt* 2013;18:120502.
15. Wilson JW, Vajzovic L, Robles FE, et al. Imaging microscopic pigment chemistry in conjunctival melanocytic lesions using pump-probe laser microscopy. *Invest Ophthalmol Vis Sci* 2013;54:6867–76.
16. Robles FE, Deb S, Wilson JW, et al. Pump-probe imaging of pigmented cutaneous melanoma primary lesions gives insight into metastatic potential. *Biomed Opt Express* 2015;6:3631–45.
17. Loui AP, Venetsanopoulos AN, Smith KC. Morphological autocorrelation transform: a new representation and classification scheme for two-dimensional images. *IEEE Trans Image Process* 1992;1:337–54.
18. Matthews TE, Wilson JW, Degan S, et al. In vivo and ex vivo epi-mode pump-probe imaging of melanin and microvasculature. *Biomed Opt Express* 2011;2:1576–83.
19. Wilson JW, Degan S, Gainey CS, et al. Comparing in vivo pump-probe and multiphoton fluorescence microscopy of melanoma and pigmented lesions. *J Biomed Opt* 2015;20:051012.
20. Cinotti E, Perrot JL, Labeille B, et al. Reflectance confocal microscopy for the diagnosis of vulvar melanoma and melanosis: preliminary results. *Dermatol Surg* 2012;38:1962–7.
21. Debarbieux S, Perrot JL, Erfan N, et al. Reflectance confocal microscopy of mucosal pigmented macules: a review of 56 cases including 10 macular melanomas. *Br J Dermatol* 2014;170:1276–84.
22. Agozzino M, Buccini P, Catricalà C, et al. Noninvasive assessment of benign pigmented genital lesions using reflectance confocal microscopy. *Br J Dermatol* 2015;173:1312–5.
23. Yélamos O, Merkel EA, Sholl LM, et al. Nonoverlapping clinical and mutational patterns in melanomas from the female genital tract and atypical genital nevi. *J Invest Dermatol* 2016;136:1858–65.

JOURNAL OF
LOWER
GENITAL TRACT DISEASE

Be Social.

Connect with the Journal of Lower Genital Tract Disease.

Visit us on **Facebook**: <http://www.facebook.com/JLGTD>

Follow us on **Twitter**: <http://twitter.com/JLGTD>

Join our **LinkedIn** group: <http://www.linkedin.com> [Groups: Journal
of Lower Genital Tract Disease]

The Journal of Lower Genital Tract Disease, is sponsored by the American Society for Colposcopy and Cervical Pathology, the International Society for the Study of Vulvovaginal Disease, the Australian Society for Colposcopy and Cervical Pathology, the Society of Canadian Colposcopists, and the International Federation of Cervical Pathology and Colposcopy. The Journal is dedicated to the study and interchange of ideas regarding health and diseases of the lower genital tract.