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Validation of noninvasive focal depth measurements to determine epithelial thickness of the vaginal wall

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Abstract

Objective: This study investigates whether noninvasive focal depth (FD) measurements correlate with vaginal wall epithelial thickness (ET). If FD accurately reflects ET of the vaginal wall, this would allow noninvasive longitudinal assessment of (newly developed) treatment modalities aiming to increase ET, without the need for invasive biopsies.

Methods: Fourteen women, median age 62 years (inter quartile ranges: 57-65), undergoing vaginal prolapse surgery because of anterior and/or posterior compartment pelvic organ prolapse were included. We used the CytoCam, a handheld video microscope based on incident dark field imaging, and performed FD measurements of the vaginal wall before surgery. Histology was performed on tissue that was removed during the surgical procedure, and ET was measured in stained sections. We compared ET with FD interindividually, and determined the expected linear correlation and agreement between the two measurements.

Results: Seventeen ET measurements (mean 125 μm ± 38.7, range 48-181 μm) were compared with 17 FD measurements (mean 128 μm ± 34.3, range 68-182 μm). The lineair correlation between the two measurements was strong ($r = 0.902$, $P < 0.01$). Bland-Altman analysis demonstrated a mean difference of 13.5 μm when comparing ET to FD.

Conclusions: The results demonstrate good agreement between ET and FD measurements. We consider the mean difference demonstrated with Bland-Altman analysis acceptable for these measurements. This suggests that FD accurately reflects ET, which further supports the use of FD to measure ET of the vaginal wall. For a complete assessment of the vaginal wall, FD measurements are preferably combined with the assessment of vaginal angioarchitecture.

Key Words: CytoCam – Epithelial thickness – Focal depth – Microcirculation.

Vulvovaginal atrophy (VVA) is a common ageing condition, affecting an estimated 40% of postmenopausal women.[1] As a consequence of vaginal discomfort and pain, VVA has significant impact on daily activity, sexual function, and overall quality of life.[2] The diagnosis and evaluation of treatment are predominantly done by clinical assessment.[3] Objective measures such as the vaginal maturation index or vaginal pH are either expensive and time-consuming or inaccurate in the case of the latter.[4] Clinicians and researchers would benefit from a method that accurately and directly measures VVA, for objective assessment and evaluation of (newly developed) treatment modalities.

As VVA is characterized by a thin epithelium, measuring epithelial thickness (ET) has been suggested as a method.[4,5] Previous histological studies have demonstrated that ET increases after treatment with topical estrogen,[6,7] and possibly also after vaginal laser therapy.[8] Histology, however, requires invasive tissue sampling and is therefore not easily applicable in a daily clinical or research setting.

In a previous study, it was suggested that ET can be measured in vivo and noninvasively with the use of incident dark field (IDF) imaging.[5] This technique is primarily used to investigate the microcirculation.[9-11] It uses green light that is absorbed by hemoglobin in red blood cells, allowing visualization of the microcirculation.[9] In addition, the IDF-device records the distance from the tip of the device to the point

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AWK and CMD equally contributed to this work.

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where the microcirculation is in optimal focus, defined as focal depth (FD). Because the vaginal vasculature is located in the subepithelial layers of the vagina (lamina propria) and the epithelium is not directly vascularized, FD is considered to be a reflection of ET.[5]

It was demonstrated that FD increases significantly after application of topical estrogen,[5] but it is unknown whether FD measurements reflect the actual thickness of the epithelium. Therefore, we compared vaginal wall FD measurements with ET measured in participant-derived tissue samples from imaged sites, and determined correlation and agreement between the measurements. A good correlation between these two measures would further rationalize the use of FD and justify replacement of histology by noninvasive FD measurements, not only for VVA, but in many other (fibrotic, sclerosing) conditions that affect the epithelium. In case of VVA, this would allow noninvasive, longitudinal assessment of (newly developed) treatment modalities aiming to increase ET.

MATERIALS AND METHODS

Participants and settings

In this prospective, single-center observational study, we recruited women with (1) pelvic organ prolapse stage 2 or more, (2) undergoing anterior and/or posterior colporrhaphy at the Department of Obstetrics and Gynaecology who (3) could be informed about study procedures and guidelines in their native language. Medical history was obtained and women with history of vaginal surgery were excluded from participation. Women received verbal explanation of the study and written informed consent was obtained from each participant. All approached women ($n = 14$) agreed to participate in our study. This study was reviewed by the Medical Ethics Committee under number W17_040, and permission was granted on February 2, 2017.

Focal depth measurements

Focal depth measurements were performed using incident dark field (IDF) imaging (CytoCam, Braedius Medical, Huizen, The Netherlands).[9] The CytoCam is a commercially available hand-held video microscope, predominantly used for assessment of the sublingual microcirculation in critically ill patients. In addition, the CytoCam has been used for assessment of the microcirculation of different organ surfaces, including the vagina. CytoCam measurements are noninvasive and safe: no adverse events or complications have been reported as a consequence of measurements performed with the CytoCam. In previous studies, vaginal measurements were well tolerated by study participants. The CytoCam is lightweight (120 g) and shaped like a pen (length 220 mm, diameter 23 mm), and uses green light, emitted from high-brightness LEDs located at the tip of the device. Green light is absorbed by hemoglobin in the red blood cell, allowing visualization of the microcirculation. The CytoCam measures the distance from the tip of the device to the point where the microcirculation is in optimal focus (ie, when individual erythrocytes can be discriminated). In practice, this is the distance between the subepithelial microcirculation and the epithelial surface, in micrometers. The CytoCam can focus between 0 and 400 μm and the focus can be adjusted with steps of 1 μm. The CytoCam was connected to a device controller which, in turn, was connected to a powerful laptop (P50, Lenovo, Peking, China) that was used for visualization and storage of the images. All measurements were performed on participants in the operating room before surgery by one researcher (AK) experienced in measuring the vaginal microcirculation and performing FD measurements. All measurements were performed while patients were under general anesthesia. The CytoCam was placed into contact with the mucosa of the vaginal wall without applying excessive pressure, at 3 cm above the hymen at the surgical site. Subsequently, the CytoCam was adjusted for optimal focus. Optimal focus was acquired in micrometers for the most superficially located blood vessels (ie, the smallest value at which the microcirculation was in focus, was noted as the focal depth). In all participants, the complete measurement took less than 3 minutes.

Tissue retrieval and histological assessment of epithelial thickness

After hydrodissection with a saline solution with 24% adrenaline, a midline incision of the vaginal wall tended by four Allis clamps was performed. After plication, excessive and redundant vaginal tissue was trimmed and removed. We avoided clamping of the imaged part of the redundant tissue, in order not to manipulate the epithelium. From the removed tissue, a tissue sample of approximately 10 × 10 mm was taken, which was formerly located 3 cm above the hymen. Tissue samples were fixed in 10% neutral-buffered formalin immediately after harvesting, for 48 hours. After fixation and dehydration, tissue samples were embedded in paraffin, cut into 5 μm thick sections and stained with hematoxylin and eosin (H&E) staining (Benchmark ULTRA, Roche Diagnostics, Indianapolis, IN) for histological analysis. Stained sections were analyzed by light microscopy and images were acquired using the Olympus cellSense Standard software (Olympus, Leiderdorp, The Netherlands) and an Olympus BX51 microscope (Olympus) with ×20 objective. In the acquired images, ET (ie, stratified squamous epithelium, superficial layer until lamina propria) was measured with ImageJ software (ImageJ, NIH and LOCI, University of Wisconsin, Madison, WI).[12] From each section, ET was measured at crypts, where the distance from the surface of the vaginal wall to the vasculature in the lamina propria was the smallest. When no crypts were present, the thinnest measure of ET in that sample was used. Measurements of ET were performed by two individual researchers (AK, CD) who were blinded for sample identity, and measurements were averaged.

Statistical analysis

The primary outcome was the agreement between the two measurement techniques. The thinnest ET measured in sections was the reference measurement. Descriptive statistics were used for data summaries, that is, a mean with SD when

normally distributed and median with inter quartile ranges (IQRs) when not normally distributed. We used a d'Agostino and Pearson test to determine distribution of data. The correlation between ET in histological slides and FD measured with the CytoCam was examined as r by calculating the Pearson correlation coefficient (PCC). A correlation coefficient of 0.9 to 1.0 will be considered a very high positive correlation, 0.7 to 0.9 as a high positive correlation, 0.5 to 0.7 as a moderate positive correlation, and so on.[13] A minimal sample size of 13 participants was determined for a correlation coefficient of 0.7, with the alpha level set at 0.05 and the beta level set at 0.20. The level of agreement and the 95% limits of agreement between the two measurements were determined using the Bland-Altman method.[14] All results were analyzed in SPSS Statistics 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.).

RESULTS

Fourteen women provided written and verbal informed consent and were included in this study. The median age was 62 years (IQR 57-65) and participants had no relevant comorbidity (ASA I-II). All participants were operated for an anterior and/or posterior compartment pelvic organ prolapse, POP-Q stage 2 or more. In total, 17 locations were imaged: 13 measurements of the anterior vaginal wall, and 4 measurements of the posterior vaginal wall. Three women were operated for both an anterior and posterior compartment prolapse, in which case both the anterior vaginal wall and the posterior vaginal wall were measured.

Focal depth and epithelial thickness measurements

Focal depth could be determined in all participants. Different types of micovascular morphology, also known as angioarchitecture,[10] were observed during FD measurements (Fig. 1). The mean FD measured with the CytoCam in all participants was 128 μm (SD 34.3 μm, range 68-182 μm) (Table 1). In all HE-stained sections, a normal structure of vaginal tissue was observed, including the epithelium, the lamina propria, a muscularis layer, and the adventitia.[15] The mean ET measured in all histological samples was 125 μm (SD 38.7, range 48-181 μm) (Table 1). Within a section, ET was often heterogeneous. Figure 2 illustrates a histological sample with heterogeneous ET ranging from 160 to 320 μm. In this case, the thinnist measure of ET in that sample (160 μm) was used for comparison.

TABLE 1. *Overview of study participants and the performed measurements*

Participant number	Histology epithelial thickness (μm)	CytoCam focal depth (μm)
1	160	160
2	83	100
3	174	170
4	115	92
5	154	156
6	126	128
7	119	106
8	160	182
9 A	110	124
9 B	48	83
10 A	134	124
10 B	86	68
11	181	172
12	123	127
13 A	168	157
13 B	65	93
14	119	137
$\dot{X}$	125	128
SD (range)	38.7 (48-181)	34.3 (68-182)

Histology measurements of epithelial thickness were performed in participant-derived tissue samples and values are expressed in micrometers. Focal depth measurements were performed with the CytoCam; values are also expressed in micrometers. Mean ($\dot{X}$), SD, and range of the measurements are displayed as well.

Correlation and agreement between measurements

Comparison of ET with FD demonstrated a very high positive correlation ($r = 0.902$, $P < 0.01$) (Table 2). Figure 3 graphically displays this positive linear relationship between the two measurements. The Bland-Altman plot in Figure 4 shows the level of agreement between the two measurements and the 95% limits of agreement. This analysis demonstrated that the mean observed difference between the two measurements was 13.5 μm with a 95% CI of −5.9 to 32.9 μm.

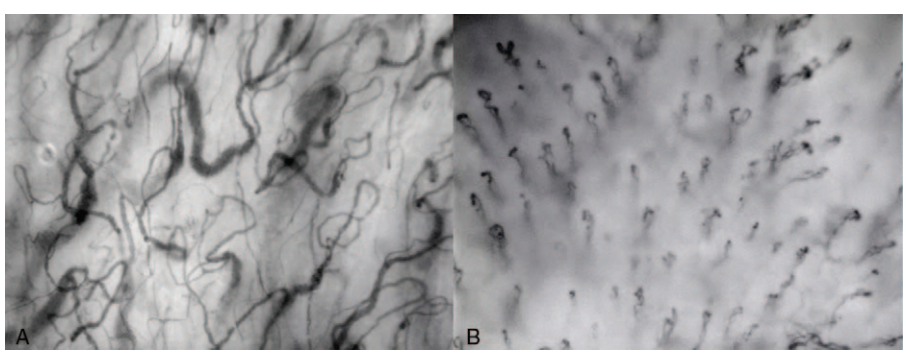

FIG. 1. Screenshots of CytoCam images with different types of angioarchitecture. Each image represents an area of 1.55 × 1.16 mm. (**A**) The focal depth is 80 μm. (**B**) The focal depth is 160 μm.

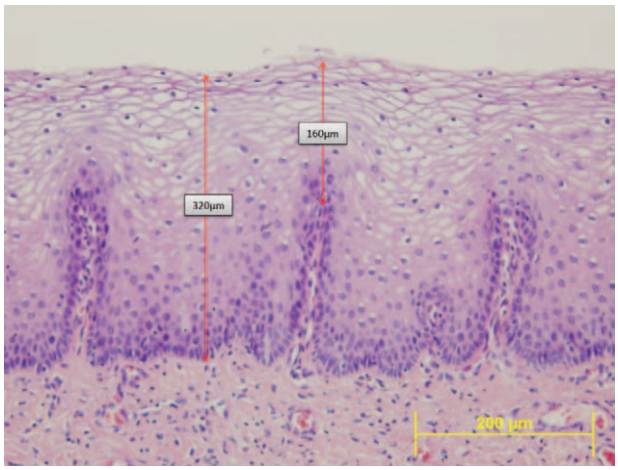

FIG. 2. Hemotoxylin and eosin (H&E) staining of the vaginal wall. The epithelial thickness is heterogeneous and ranges from 160 to 320 µm. In this participant, the CytoCam focal depth was 160 µm.

DISCUSSION

Main findings

The results of this study suggest that FD measurements have a strong correlation with vaginal wall ET. We have demonstrated that FD accurately reflects the thinnest measure of ET within an individual.

Strenghts and limitations

The study design allowed us to acquire vaginal tissue for histological analysis and ET measurements, without the need for additional invasive procedures. Double, independent, and blinded ET measurements were performed in sections and compared with FD measurements. Because no strict inclusion and exclusion criteria were used, the possibility for sampling bias was reduced.

This study also has several limitations. A relatively small sample size was used, which increases the possibility that our findings are based on chance. Also, all participants were women with pelvic organ prolapse, which makes our sample a specific selection of women. The fact that participants had no relevant comorbidity and a median age of 62 years increases the generalizability of this sample to the broader population with VVA.

Limitations with respect to the measurements have to be considered as well. We did not mark the location that was imaged. Therefore, the selected tissue sample might not exactly be the imaged location. By standardizing the FD measurements as much as possible (midline, 3 cm above the hymen), we, however, limited this chance. In addition, we determined ET measurements in sections as the gold standard. There are, however, some factors that could affect the thickness of the epithelium in sections, or the way it is measured. First, fixation in formalin can hypothetically alter the dimensions of tissue.[16] Nevertheless, research in other tissues demonstrated that these changes are not observed or are very limited.[17,18] Second, when tissue samples are embedded and subsequently sectioned, sectioning is never performed in an exact 90° perpendicular position. The less this 90° position is respected and the more oblique the section is made, the thicker the epithelium will appear in a section.[19] Third, placement of Allis clamps and traction on the tissue could affect the dimensions.

There are also factors that can affect FD measurements. First, FD is a subjective measure, in which the user has to determine the optimal focus of the camera. Because of practical reasons, only one trained investigator performed the FD measurements, which would ideally have been done by two independent researchers. As also mentioned for ET in sections, a non-90° position of the CytoCam would hypothetically also affect FD and a 90° position cannot be guaranteed.

Interpretation of results

The vaginal wall is composed of four layers: the epithelium, the lamina propria, a muscularis layer and the adventitia.[15] The epithelial layer is not directly vascularized, but is provided with oxygen via diffusion from the vasculature in the subepithelial layers.[19] The fact that the epithelium is avascular provides the rationale for measuring FD by using the distance from basal membrane to vascular netwerk as proxy for ET.[5] VVA is characterized by a thin epithelium, whereas a thick epithelium is observed in women without VVA.[20] In previous studies that evaluated ET in biopsies, the reported ET ranged from 200 to 2.5 mm.[21] In our study, the ET measured in tissue samples ranged from 48 to 405 µm, with great intraparticipant-variation. The large variation within participants can be explained by crypts and rugae, causing the epithelium to fold strongly. Figure 2 demonstrates ET ranging from 160 to 320 µm in one tissue sample. In this case, the FD measured by the CytoCam was 160 µm. This demonstrates that FD reflects the thickness of the epithelium where the capillary bed is most superficial (ie, the thinnest part of the epithelium). In the present study, FD measurements ranged from 68 to 182 µm, which is within the same range as our previous study.[5] It should also be noted that the imaged area with the CytoCam is aproximately 1.8 mm^2, indicating that FD is an avarage of that area, from which only a small region is measured in a section. Interestingly, the epithelium of the posterior vaginal wall was thinner in all participants than the epithelium of the anterior vaginal wall.

Clinical implications

Currently, measurements of ET are mainly performed in a research setting to evaluate new treatment modalities for VVA, such as vaginal laser therapy. This study further supports the use of noninvasive FD measurements to replace these invasive, histological measurements of ET. Although

TABLE 2. *Mean difference (Ẋ) and SD in micrometers between measurements of epithelial thickness and focal depth*

	Ẋ	SD (range)	Pearson *r*
Δ Histology – CytoCam	13.5	9.9 (0-35)	0.9

Correlation is determined as Pearson *r*.

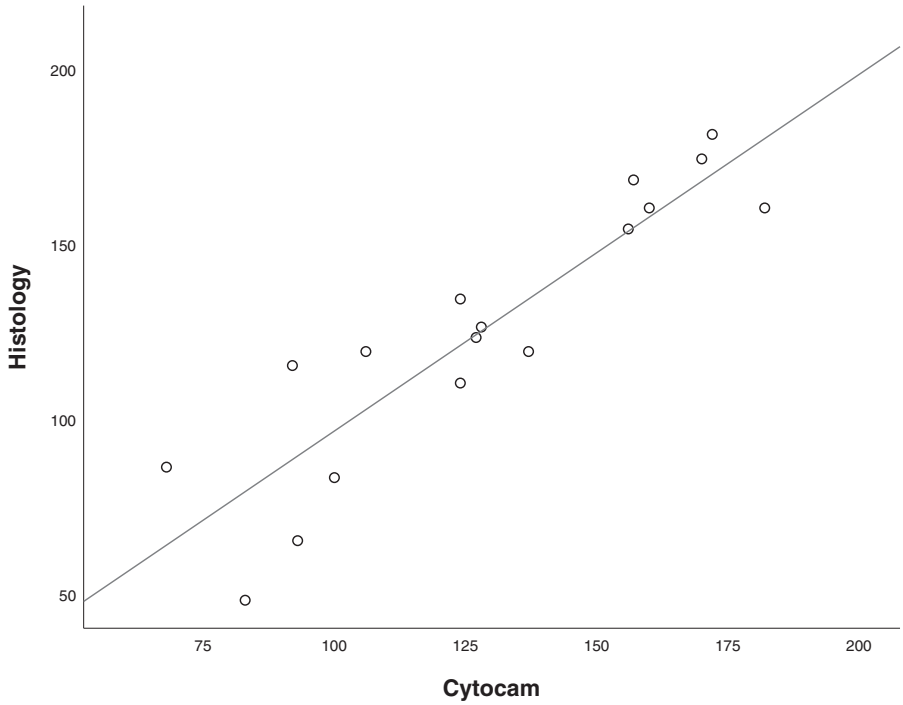

FIG. 3. Scatterplot comparing epithelial thickness (histology) to focal depth (CytoCam).

we have now demonstrated that FD measurements underestimate the *mean* ET, our previous study demonstrated that significant changes in FD after treatment could be measured. This suggests that not only the *mean* ET increases after treatment, but that the thinnest measure of ET increases as well. It should, however, be noted that the true increase of (mean) ET might be more significant than is measured with FD.

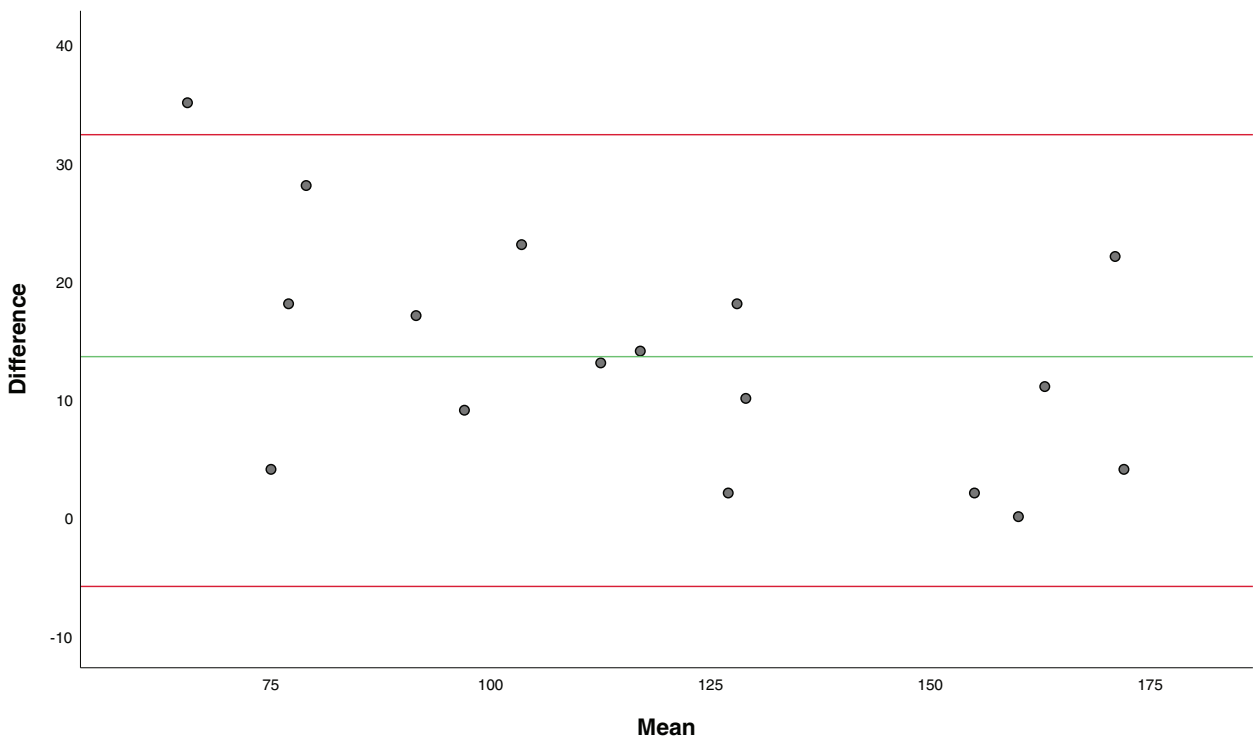

FIG. 4. Bland-Altman plot of the difference between epithelial thickness (histology) and focal depth (CytoCam).

To perform a more complete and even more reliable assessment of the vaginal wall, FD can be combined with assessment of the vaginal angioarchitecture (ie, structure and layout of vascular network). In a previous study, we demonstrated that an altered angioarchitecture is observed in women with VVA.[22] In these participants, we observed a loss of capillary loops, and appearance of a vascular network. We also demonstrated that estrogen has the potential to restore these alterations. When combining FD measurements and assessment of angioarchitecture, a more complete assessment of the vaginal wall can be performed. We argue that treatment for VVA should not only increase ET, but also improve vaginal angioarchitecture.

We suggest that CytoCam-IDF imaging can be used in research as well as in clinical practice. For a broader application in clinical practice, the costs and cost-effectiveness of this method would have to be analysed. The relatively expensive one-time purchase of a CytoCam system would have to be balanced against the advantages of this method, such as direct and accurate diagnosis of VVA. This could be a subject of future research.

CONCLUSIONS

Focal depth measurements can be used as an accurate and noninvasive measure of epithelial thickness. Focal depth measurements reflect the thinnest measure of the epithelium within an indivual.

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