Published in final edited form as:

Obstet Gynecol. 2011 June ; 117(6): 1307–1313. doi:10.1097/AOG.0b013e31821c33dc.

Differences in Primary Compared With Secondary Vestibulodynia by Immunohistochemistry

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Abstract

Objective—To assess whether primary and secondary vestibulodynia represent different pathologic pathways.

Methods—This was an analysis of archived vestibulectomy specimens from 88 premenopausal women with vestibulodynia (2002–2008). Patient records were reviewed to classify the type of vestibulodynia, duration of symptoms, and hormone status. Histologic sections were stained for hematoxylin and eosin to grade inflammation, S100 to highlight nerves, CD117 for mast cells, estrogen receptor α , and progesterone receptor. Differences between primary and secondary vestibulodynia were tested by t-tests, chi-square analysis, linear and logistic regression.

Results—Primary vestibulodynia showed significant neural hypertrophy and hyperplasia ($p=0.02$, adjusted odds ratio 3.01 [1.2–7.6]) and increased progesterone receptor nuclear immunostaining ($p=0.004$, adjusted odds ratio 3.94 [1.6–9.9]) compared with secondary vestibulodynia. Estrogen receptor α expression was also greater in primary vestibulodynia when symptom diagnosis was less than 5 years ($p=0.004$, adjusted odds ratio 5.53 [1.71–17.91]).

Conclusions—Primary and secondary vestibulodynia have significantly different histologic features, suggesting that they may have separate mechanistic pathways. Clinically, this may mean the discovery of distinct conditions.

INTRODUCTION

Vestibulodynia is a localized vulvar pain condition that commonly results in entry dyspareunia.^{1–2} Although the cause of vestibulodynia remains unclear, histologic evaluation of the vulvar vestibule tissues of affected women has revealed that the skin has more and larger nerves compared to this tissue in unaffected women. Researchers speculate that this change is in part or fully responsible for the manifestation of pain.^{3–5} Although a lymphocytic infiltrate has been found to occur locally within painful tissue^{3–6}, a classic

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Presented at the 2010 meeting of the Pacific Coast Obstetrical and Gynecological Society, Kona, Hawaii, October 1, 2010. Portions of these results were also presented at the 2010 United States and Canadian Academy of Pathology, Washington, DC, March 22, 2010.

Financial Disclosure: The authors did not report any potential conflicts of interest.

inflammatory response has not been found^{7–10}, and treatments that target the cyclo-oxygenase 2 or nitric oxide systems are likely to be ineffective.¹¹ Mediators of this neuro-inflammatory response, such as hormonal triggers (estrogen, progesterin), infection and inflammatory factors (mast cells, heparanase) continue to be investigated.^{12–19}

In a prospective study of 24 premenopausal subjects²⁰, our research team investigated hormonal and histologic markers in subjects with vestibulodynia compared to subjects without the condition (controls). Affected women were grouped into two types of vestibulodynia: primary or secondary. Primary sufferers experienced pain at first introital touch, whether with tampon insertion or sexual debut. Women with secondary vestibulodynia experienced pain after an interval of painless intercourse. Similar to previous reports, we found more neural hyperplasia/hypertrophy and inflammation in vestibulodynia specimens compared to negative control biopsies, but we also observed a trend towards more neural hyperplasia/hypertrophy and less chronic inflammation in primary vestibulodynia (n=10) compared with secondary disease (n=10).

Our prior study raised a few questions. First, it suggested the mechanistic pathways in primary and secondary vestibulodynia may be different. Therefore, if we control for disease duration, will primary and secondary vestibulodynia have similar or different histopathology? Second, if they do represent distinct pathways, what insights will histopathology provide? We now present a larger analysis of our archived vestibulectomy specimens to address these questions.

MATERIALS AND METHODS

Samples

Using an IRB approved protocol (IRB#3613 Oregon Health & Sciences University), we identified 88 cases of premenopausal vestibulodynia in our Oregon Health & Science University (OHSU) Pathology Database (2002–2008) by matching for diagnosis (vulvar vestibulitis syndrome, vulvodynia, vestibulodynia) or procedure (vestibulectomy). We searched billing records by procedure code and cross-checked MG's personal database of all vestibulectomy surgeries. Archived tissue specimens were obtained from patients previously seen in the OHSU Program in Vulvar Health that met Friedrich criteria for vestibulodynia²¹ and had severe or moderate dyspareunia. All clinic visits and outpatient vestibulectomies were performed by either CML or MFG (authors). All specimens were obtained from localized vestibulectomies in which the hymen and occasionally 1cm of distal vagina was used as the surgical flap.²² Patient clinical records were reviewed by CML and MFG to verify patient age, parity, premenopausal status, primary versus secondary classification, hormonal status (combined contraceptive steroids and menstrual cycle phase), and duration of symptoms.

Histopathology

Routine paraffin-embedded histologic sections (3–5µm) were stained for hematoxylin and eosin (H&E) to grade for inflammation. Serial sections were immunostained for S100 (nerves) (DAKO, Carpinteria, CA), CD117 (mast cells) (DAKO), presence of progesterone receptor (PR) (DAKO), and presence of estrogen receptor α (ER α) (DAKO) as we previously described in our prospective study.²⁰ Notably, ER β and epidermal nerve twig staining with PGP9.5 were not informative in our prior study²⁰ and were therefore not used in this retrospective analysis. Staining quality and specificity was confirmed by an expert pathologist (TMK) using appropriate controls (e.g. nerves for S100 and breast tissue for hormone receptors).

Histopathologic Semiquantitative Analysis

Cases were independently scored by two pathologists (TKM and VBK) who were blinded to clinical and demographic data. Scoring was performed as described in our prior study.²⁰ Briefly, chronic inflammation was scored as none (0), mild (1+) or moderate (2+). S100 positive nuclear immunostaining in nerves was scored as none (0), mild (1+), moderate (2+), or severe (3+) neural hypertrophy/hyperplasia. Estrogen and progesterone receptor nuclear immunostaining were scored similar to routine breast pathology using a 0–3+ scale: 0= negative; 1+= <10%; 2+= 11–50%; 3+= 51–100% of basal cells. CD117 positive mast cells were quantified as the average number of mast cells in the three high power microscopic fields (40× objective) with the most mast cells in the biopsy. Significant discrepancies between pathologists' scores were settled by joint arbitration and mast cell counts provided by each pathologist for each case were averaged.

Statistical Analysis

Statistical analyses of the data were performed with SAS statistical software (SAS Institute Inc, Cary, NC). Histology agreement between pathologists is reported via the Kappa statistic. Comparisons of patient characteristics between primary and secondary vestibulodynia were conducted using chi-square tests for dichotomous variables and t-tests with Satterthwaite adjustment for unequal variances for continuous variables.²³ Histologic findings were compared by proportional odds logistic regression models and ordinary binary logistic regression.²⁴ The proportional odds logistic regression models take advantage of the ordinal nature of the outcomes and are more powerful than binary logistic regression for a given sample size.²⁵ Linear regression analyses were used to compare mean number of mast cells in primary and secondary vestibulodynia. Age and duration were incorporated into logistic and linear regression models after application of the natural logarithmic transformation (due to skewness). We present, separately, results adjusted for age and duration then results adjusted for age, duration, combined contraceptive steroid use, and parity. In the discussion of results we focus on the full models that adjust for age, duration, combined contraceptive steroid use and parity, and refer to the associated estimated odds-ratios (mean differences) as fully adjusted odds-ratios (mean differences). Because the cell sizes were not large, we compared results to models adjusting for confounders one at a time as well as results from models in which the four-category ordinal outcomes were collapsed to two categories.

RESULTS

Forty-two of the 88 cases had primary vestibulodynia (48%) and 46 had secondary (52%). The number of premenopausal women on combined contraceptive steroids was nearly equal in the primary (52%) and secondary (54%) groups. Similar to prior reports¹⁹, patients with primary vestibulodynia were significantly younger (median = 26.5 years) than women with secondary vestibulodynia (median = 29.5 years, p 0.004) with the median age of all subjects 28 years. Primary cases tended to be nulliparous compared to women with secondary vestibulodynia (83% compared with 67%, respectively, p 0.08); and, symptom duration was significantly longer in primary (median = 6 years) versus secondary disease (median = 4 years, p 0.002).

It is noteworthy that the majority of cases had vestibulodynia for years before having a vestibulectomy. Women reported trying multiple therapies for vestibulodynia before a vestibulectomy. Of the 46 women with secondary vestibulodynia, 57% recalled associations with particular health events: 22% related that the onset of pain was postpartum; 17% recalled that pain began within months (<3) of treatment for HPV by LEEP or laser; 11% stated pain began after an episode of complicated cystitis; 4% noted an unusually severe

vaginitis when pain started and 2% reported an irritable bowel syndrome flare associated with onset of pain.

We observed good to excellent agreement between pathologists scoring histologic sections for inflammation, neural hypertrophy/hyperplasia, and hormone receptors (kappa statistics ranged from 0.63 to >0.8). Similar to our prior prospective study²⁰, we observed significantly more neural hypertrophy and hyperplasia (Figure 1; Figure 2 A–B) in primary vestibulodynia compared with secondary (Table 1). The significant difference persisted even after adjusting for age, parity, combined contraceptive steroid use, and duration of symptoms (fully adjusted odds ratio (OR) = 3.01, $p=0.02$).

There was no difference in chronic inflammation between primary and secondary cases (unadjusted OR 0.65, $p=0.31$; fully adjusted OR 0.64, $p=0.37$). In addition, none of the potential covariables (age, parity, duration of symptoms and combined contraceptive steroid use) were associated with the level of inflammation. Mast cell numbers were increased in both types of vestibulodynia (Figure 1), but there was no difference between primary and secondary (fully adjusted mean difference 0.49, $p=0.85$).

Secondary vestibulodynia showed less progesterone receptor (PR) staining than primary disease (Figure 1; Figure 2 C–D). PR receptor density was significantly greater in primary vestibulodynia by both univariable and multivariable analyses (fully adjusted OR 3.94, $p=0.004$) (Table 1). Examination of multivariable models for ER expression suggested potential differences between primary and secondary conditions. In women with symptom recognition less than 5 years, the primary group was more likely (fully adjusted OR 5.53, $p=0.004$) than the secondary group to have more ER (Figure 1; Figure 2 E–F). With longer symptom duration there was no significant difference in ER expression (fully adjusted OR 0.69, $p=0.59$) between primary and secondary disease. When dichotomizing ER and PR (collapsing the two smallest and two largest categories), we found results that were very similar to those described in Table 1. In particular, for PR, which had a marked change from unadjusted to adjusted analyses, the corresponding ORs based on the dichotomous outcome were 2.77 ($p=0.02$), 3.61 ($p=0.01$) and 3.63 ($p=0.02$) for the unadjusted model, the model adjusting for age and duration, and the model adjusting for age, duration, combined contraceptive steroid use and parity, respectively. Also, we note that both age and duration appear individually to be negative confounders in the relationship between primary/secondary status and PR. When adjusting for age and duration individually the ORs for PR are 3.17 ($p=0.007$) and 3.14 ($p=0.007$), respectively. The similarity of the results from the different adjusted proportional odds models and the similarity of the results from binary logistic regression to those based on using the full set of categories suggest that the results are fairly robust even with the small sample size.

DISCUSSION

Our data support the hypothesis that primary and secondary vestibulodynia may have distinct histopathologic pathways. Both subtypes showed increased chronic inflammation with mast cell recruitment. However, primary type showed significant neural hypertrophy and hyperplasia with increased progesterone receptors compared to secondary. These differences persisted even after controlling for disease duration, which suggests that primary and secondary vestibulodynia may develop along different pathogenic pathways, rather than representing different stages of the same disease.

Previous studies have demonstrated neural hypertrophy and hyperplasia in vestibulodynia compared to negative controls^{3–6}, but those smaller studies did not distinguish between primary and secondary vestibulodynia. Our prior prospective analysis found significant

differences between these two clinical variants²⁰, but it too lacked the statistical power to reliably test this hypothesis. For example, in our prior study, we observed more chronic inflammation in secondary vestibulodynia than in primary.²⁰ This larger study does not support that observation, showing equivalent increases of chronic inflammation in both primary and secondary groups. Therefore, chronic lymphocyte infiltrates may be integral to maintaining the pain process in both subtypes^{7–10, 13–14, 18}, and in fact, neurotrophins and inflammatory mediators have been implicated in abnormal nociceptive function.^{26–27} Moreover, in our prior prospective study, we observed increased chronic inflammation in both the tender and *non-tender* areas of the vestibule, suggesting that the entire vestibule demonstrates hallmarks of an altered process.²⁰ We conclude that the neuro-inflammatory relationship in vestibulodynia deserves continued investigation.

Endocrine causation has been implicated in the development of vestibulodynia, and our data revealed hormonal receptor differences between primary and secondary vestibulodynia. Loss of estrogen has been hypothesized as a trigger for some women with vestibulodynia.^{16–17} Clinically, lack of estrogen provokes vestibulodynia in postpartum and postmenopausal women.^{28–29} Of our 46 women in the secondary group, 22% developed pain in a postpartum setting, consistent with an earlier descriptive series on vestibulodynia.³⁰

Case control studies have suggested that oral contraceptive use is associated with vestibulodynia.^{31–34} This is an important clinical issue due to the widespread use of combined contraceptive steroids. Clinically, very few women note variations in their vestibular pain associated with these medicines or the menstrual phase.³⁰ Combined contraceptive steroids are progestin dominant and although progestins are known to down-regulate PR³⁵, this has not been demonstrated in vestibule tissues.³⁴ Also, Johansson's small controlled study evaluating women on and off combined contraceptive steroids noted only minor differences in vestibular ER receptor status.³⁴ Our data, too, did not demonstrate receptor differences in women on combined contraceptive steroids compared to non users.

Our study may provide insights into different potential triggers and associations for primary and secondary vestibulodynia but it does not prove causation. For example, our data suggest that the loss of PR is associated with secondary vestibulodynia. Thirty-two percent of secondaries identified infection as a trigger, but how this might relate to PR is unclear. The trigger for primary vestibulodynia is not clinically evident since these patients present having had the condition from first penetrative experience, usually many years previous. However, we cannot exclude the role of estrogen in an earlier step in the development of the process. There is no data regarding normal variation of introital tenderness in prepubertal girls and it is possible that childhood tenderness from low estrogen resolves with the pubertal rise in estrogen. If the rise in pubertal estrogen locally fails in a small proportion of young women, we hypothesize that this may lead to a neuro-inflammatory response in the vestibular tissue and subsequent vestibulodynia. This remains to be tested.

Finally it is interesting to consider that vestibulodynia may be similar to interstitial cystitis, another chronic pain condition in a tissue with identical embryonic derivation (endoderm). Both interstitial cystitis and vestibulodynia are histologically characterized by chronic inflammation, increased mast cells, and neural hyperplasia/hypertrophy.³⁶ Indeed, some women have both interstitial cystitis and vestibulodynia.³⁷ Moreover, interstitial cystitis also appears to be related to an ER/PR imbalance.³⁸

This large study of 88 archived vestibulectomy specimens demonstrated histopathologic differences between primary and secondary vestibulodynia. Although one strength of this study is its size, heterogeneity was also introduced by this large population. To reduce the

heterogeneity, we elected to present only the findings in premenopausal women in this report. Care was taken to gather detailed histories, but it should be noted that assigning a specific duration for primary disease was especially challenging since these women recognized their pain at varying times from first attempt at tampon use to first attempted coitus later in life. Methodologically a retrospective study is descriptive and cannot control for factors like the phase of the menstrual cycle or use of combined contraceptive steroids. Our findings could not be compared to control tissues, and indeed the literature on the vulvar vestibule lacks descriptions of normal histology.

In summary, our results suggest primary and secondary vestibulodynia may have distinct pathogenic pathways. A variety of triggers (environmental, hormonal, infectious) may lead to this condition, but future studies aiming to identify and treat these triggers should discriminate between primary and secondary vestibulodynia.

Acknowledgments

Funded by the National Vulvodynia Association. Dr. Morgan's contribution was also funded by the Office of Research on Women's Health and the National Institute of Child Health and Human Development, Oregon BIRCIH HD043488-08.

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Figure 1.

Histopathology of primary and secondary vestibulodynia. Data are presented as the mean score $\pm$ standard error of the mean (SEM) for chronic inflammation, neural hyperplasia, hormone receptor expression, and mast cell number per high powered field. Multivariable analysis suggests mean symptom duration less or greater than 5 years may reveal significant differences between groups.

Figure 2.

Photomicrographs of differences between primary and secondary vestibulodynia. (A) Primary disease reproducibly showed significantly more neural hypertrophy/hyperplasia (arrows) compared to (B) secondary vestibulodynia. Progesterone receptor nuclear immunostaining (arrow) was increased in the epithelial layer of primary disease (C), but only rare positive nuclei (arrow) were seen in secondary vestibulodynia (D). Estrogen receptor α immunostaining was strong in the epithelium (arrows) of patients with primary disease (E) and the frequency of positive nuclei was slightly more than secondary vestibulodynia (F). Positive staining is dark brown (arrow); sections were counterstained with hematoxylin. All photomicrographs were taken using the 10x objective (Leica, Wetzlar, Germany).

Table

Odds Ratios and Mean Differences for Primary compared to Secondary Vestibulodynia

N=88	<u>Unadjusted OR [95% CI]</u> Primary versus Secondary	<u>Adjusted OR [95% CI]</u> Primary versus Secondary Adjusting for age and duration	<u>Adjusted OR [95% CI]</u> Primary versus Secondary Adjusting for age, duration, combined contraceptive steroids and parity
Neural Hyperplasia P ^I : 10,7,25 ; S ^I : 16,17,13	2.75 [1.23–6.14] p=0.01	3.15 [1.26–7.91] p=0.01	3.01 [1.20–7.58] p=0.02
PR P: 5,14,15,8; S:13,19,10,4	2.76 [1.26–6.06] p=0.01	4.02 [1.61– 10.03] p=0.003	3.94 [1.57– 9.89] p=0.004
ER alpha P: 2,1,6,9; S:10,10,9,6	Symptom Duration ≤ 5 years 5.14 [1.68–15.71] p=0.004	Symptom Duration ≤ 5 years 5.39 [1.68–17.33] p=0.005	Symptom Duration ≤ 5 years 5.53 [1.71–17.91] p=0.004
P: 9,5,7,3; S:4,1,4,2	Symptom Duration > 5 years 0.72 [0.20–2.65] p=0.63	Symptom Duration > 5 years 0.74 [0.20– 2.70] p=0.65	Symptom Duration > 5 years 0.69 [0.19– 2.57] p=0.59
Inflammation P:20,22; S: 17, 29	0.65 [0.28, 1.51] p=0.31	0.65 [0.25, 1.72] p=0.39	0.64 [0.24, 1.71] p=0.37
	<u>Mean Difference</u> <u>[95% CI]</u> Primary minus Secondary	<u>Adjusted Mean Difference</u> <u>[95% CI]</u> Primary minus Secondary Adjusting for age and duration	<u>Adjusted Mean Difference</u> <u>[95% CI]</u> Primary minus Secondary Adjusting for age, duration, combined contraceptive steroids and parity
Mast cells (hpf)	−1.63 [−6.16, 2.91] p=0.48	0.53 [−4.61, 5.68] p=0.84	0.49 [−4.54, 5.53] p=0.85

^I Cell counts for outcomes; Primary (P) and Secondary (S).