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Inflammatory Markers in Vestibulodynia

David A Baker, Tatyana Peresleni and Kocis Christina*

Department of Obstetrics, Gynecology and Reproductive Medicine, Stony Brook University, USA

Abstract

Objective: The objective of this study was to investigate the cytokines secretion in patients with vestibulodynia which affects up to 16% of U.S. women. Our main goal was to find out which cytokines may serve as biological markers of this debilitating condition.

Study Design: The cytokines expression profiles in 32 patients with vestibulodynia and 26 healthy volunteers were evaluated. The vaginal rinses of all participants were analyzed for the secretion of 40 cytokines using the semi-quantitative Ray-Bio Human Inflammation Antibody Arrays, followed by densitometry and statistical analyses.

Results: IL-8 was significantly increased in vestibulodynia patients compared to controls. IL-8 was the only pro-inflammatory cytokine to be up-regulated significantly. Interleukins GM-CFS, MCSF, and IL-10 were elevated in vestibulodynia patients, although not significantly. IL-12p40 and IL-12p70 were minimally elevated. Interleukins IL-1β, RANTES, IL-2, IL-15, TGF-δ1, IL-16, TNF- α, IL-4, IL-17, IP-10,EOTAXIN-1 and -2, IL-6, IL-6sR, MCP-1, G-CFS, TIMP-2, PDGF-BB, I-309, MIP-1β, IL-1α, MIP-1δ were significantly down regulated in vestibulodynia patients *vs*. controls.

Conclusions: These findings show that IL-8, with its ability to activate neutrophil granulocytes, emerged as an undoubted marker in vestibulodynia. MCSF and GM-CFS may act together with IL-8, stimulating the production of macrophages and dendritic cells and taking part in inflammation and pain development. However, whole group of cytokines and chemokines was down-regulated in vestibulodynia patients. All of the above suggests that vestibulodynia appears to be a result of non-classical cytokine-mediated inflammatory and pain syndrome, where IL-8 appears to be the prominent marker on inflammation and pain syndrome.

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Correspondence:
David A Baker, Department of Obstetrics, Gynecology and Reproductive Medicine, Stony Brook University, 100 Nicolls Road Health Sciences Center, Department of Obstetrics, Gynecology and Reproductive Medicine, T-9, 9-030, Stony Brook, NY 11694-8091, USA, Tel: 631-444-7650;
E-mail: David.Baker@stonybrookmedicine.edu

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Introduction

Vulvodynia is a chronic pain syndrome, characterized by burning pain of the vulva that occurs in the absence of relevant visible findings or an identifiable neurologic disorder [1]. Epidemiological research indicates up to 16% of women (13 million) in the US are affected by vulvodynia, with 5% experiencing this condition before age 25 [1,2]. Vulvodynia was thought to affect only white, nulliparous women, but most large epidemiologic studies fail to demonstrate differences in disease prevalence between Caucasian and African-American women [2-4]. Vulvodynia is classified into two subgroups, generalized and localized, and further subdivided into provoked, unprovoked and mixed presentations [1]. The majority of clinical presentations are either localized provoked vulvodynia (referred to as vestibulodynia) or generalized unprovoked vulvodynia [3,5]. Vestibulodynia is a syndrome of provoked pain, localized to a specific area of the vulva, which is not explained by any other condition, persisting for more than 3 months. The exact etiology of vulvodynia remains unknown. It has not been consistently linked to candidiasis, human papillomavirus, high urinary oxalates, sexual abuse, or any specific infectious, hormonal, allergic, or inflammatory processes [6]. Vulvodynia often occurs in the context of other comorbid pain conditions [7-9], with fibromyalgia and irritable bowel syndrome being the most prevalent. Researchers have presented findings that support a possible neuro-inflammatory pathogenesis of vulvodynia, similar to other chronic pain conditions [10]. While studying vulvodynia histology, several groups reported mast cell-predominant inflammation [11-14]. Assays for pro-inflammatory cytokines/neurokines have shown inconsistent results, with some reporting an increase in vulvovaginal proinflammatory cytokines [15-19]. In our study, we investigated cytokine secretion in patients with vestibulodynia (n=32, mean age 37) and in healthy controls (n=26, mean age 35) using Ray-Bio multiplex inflammatory array assays (Ray Biotech, Inc, GA). The study was IRB approved and each patient signed a written informed consent form.

Materials and Methods

Study subjects, 33 patients diagnosed with vestibulodynia (mean age 37 years old) and 26

Table 1: Statistical analysis of cytokine expression in vestibulodynia and control patients.

A-Body	Control (n=26)	patients	Vestibulodynia patients (n=33)		t-Test	
	Density	STD	Density	STD	p-Value	p-Value, %
IL-1β	28.33	8.60	12.83	3.35	0.00107	0.1
IL-13	3.79	0.93	3.70	0.23	0.94741	94.7
RANTES	24.20	2.27	14.34	1.16	0.00030	0.0
IL-2	14.32	9.21	5.62	2.06	0.00000	0.0
IL-15	9.23	1.01	5.56	0.40	0.00186	0.2
TGF-β1	15.07	1.82	8.61	0.80	0.00270	0.3
IL-3	10.56	1.86	10.85	1.03	0.84021	84.0
IL-16	9.52	0.73	5.26	0.38	0.00216	0.2
TNF-α	9.01	1.10	6.11	0.73	0.03730	3.7
IL-4	4.92	1.74	0.80	0.12	0.01793	1.8
IL-17	3.44	0.52	1.44	0.14	0.00260	0.3
TNF-β	14.75	1.04	13.10	0.71	0.34381	34.4
EOTAXIN-1	12.86	1.56	8.29	0.69	0.00051	0.1
IL-6	7.73	0.82	1.69	0.26	0.03619	3.6
IP-10	31.17	1.35	12.74	0.56	0.00000	0.0
s TNF RI	15.20	1.62	10.11	0.90	0.09110	9.1
EOTAXIN-2	12.79	1.23	6.14	0.69	0.00034	0.0
IL-6sR	9.20	0.95	4.04	0.36	0.01578	1.6
MCP-1	24.52	1.55	16.19	0.91	0.06386	6.4
s TNF RII	15.01	1.45	10.34	1.09	0.07624	7.6
G-CSF	4.39	0.86	1.73	0.41	0.00280	0.3
IL-7	4.67	0.47	3.95	0.23	0.72672	72.7
MCP-2	4.69	0.70	3.73	0.73	0.38739	38.7
PDGF-BB	9.53	1.22	1.89	0.23	0.00000	0.0
GMCSF	4.36	0.89	5.54	1.89	0.32990	33.0
IL-8	41.02	1.94	69.90	1.52	0.00021	0.0
MCSF	13.14	2.85	15.65	4.78	0.36178	36.2
TIMP-2	39.65	1.34	14.12	0.93	0.00002	0.0
ICAM-1	10.22	1.21	6.49	1.04	0.05572	5.6
IL-10	8.40	1.11	10.27	1.12	0.46251	46.3
MIG	3.61	0.45	2.47	0.66	0.13398	13.4
IFN-γ	9.04	1.85	7.70	1.87	0.55000	55.0
IL-11	2.33	0.52	2.26	0.30	0.93379	93.4
MIP-1α	9.19	0.66	5.89	0.55	0.05197	5.2
I-309	10.46	2.55	2.22	0.69	0.00002	0.0
IL-12 p40	8.02	0.80	8.88	0.92	0.59788	59.8
MIP-1β	30.22	1.34	13.80	0.64	0.00128	0.1
IL-1α	32.21	3.05	14.72	1.63	0.00066	0.1
IL-12 p70	8.16	1.71	8.38	1.00	0.88198	88.2
MIP-1δ	8.58	3.72	1.35	0.63	0.00007	0.0

Normalized pixel density, %.

control subjects without a history of vestibulodynia (mean age 36 years old) were recruited from the Obstetrics and Gynecology (OB/GYN) ambulatory clinic. Study subjects were matched for age, race, contraceptive use and time of menstrual cycle. All were free of any vaginal inflammatory process. They were negative for Candida, culture negative for gonorrhea and chlamydia. The study was approved by Stony Brook University Subjects Committee (Institutional Review Board – IRB), and Stony Brook Committee on Research Involving Human Subjects, Category B (CORHIS B). Each participant in the study signed a written informed consent form. All participants were evaluated on not having bacterial vaginosis according to Amsel's criteria [20]. Vaginal rinses (7 ml each in deionized sterile water) were collected from all participants. Vaginal rinses were kept on ice and within an hour spun at 2800rpm for 10 min. The pellets were immediately frozen at -80°C for microbiome analyses. The supernatant was aliquoted in 430 µl samples and kept frozen at -80°C. The aliquots were later analyzed using Ray-Bio Human Inflammation Antibody Array C3 8-well plates according to the manufacturer's instructions/manual. Participant serum underwent semi-quantitative detection of 40 human proteins, and all cytokines were evaluated in duplicates. After treatment with biotinylated antibodies, followed by HRP-Streptavidin –labeled antibodies, the chemiluminescence detection of the products was performed using X-ray films (Carestream, Kodak, Biomax XAR Film, and High Performance Autoradiography). Digital images were taken by Panasonic DMC-FZ150 (res. 600 dpi). Numerical densitometry data were extracted by NIH software program Image J, v. 1.49r (http://imagej.nih.gov/ij/) using dot array analysis plugin (ROI= 45, background subtracted). Data were normalized by dividing samples' pixel density by pixel density of positive control. Statistical analysis was done by performing Student's t-Test (2 tails, unequal variances) using Microsoft Excel Analysis Tool Pack (Table 1). Bar diagrams were created using Microsoft Excel.

Results

Our study results showing varied expression of inflammatory factors in patients with vestibulodynia and healthy volunteers are presented in Figure 1, and Table 1. IL-8 was significantly increased (1.7 times, $p<0.001$) in vestibulodynia patients compared to controls (Table 1 and Figure 1a). IL-8 was the only pro-inflammatory cytokine to be up-regulated significantly (Table 1, Figure 1a and b). Interleukins GM-CFS, MCSF, and IL-10 were elevated in vestibulodynia patients 1.27, 1.2 and 1.22 times, respectively, although this increase was not significant (Table 1 and Figure 1a). IL-12p40 and IL-12p70 were also minimally elevated (1.1 and 1.02, respectively) (Table 1 and Figure 1). Interleukins IL-β, RANTES, IL-2, IL-15, TGF-β1, IL-16, TNF-α, IL-4, IL-17, IP-10,EOTAXIN-1 and -2, IL-6, IL-6sR, MCP-1, G-CFS, TIMP-2, PDGF-BB, I-309, MIP-1β, IL-1α, MIP-1δ were significantly ($p<0.005$) downregulated in vestibulodynia patients in comparison with healthy volunteers (Table 1, Figure 1a and c).

Discussion

Thus far to our knowledge, there has been no definite conclusion in the medical literature about the cytokine/chemokine profile of vestibulodynia [19]. The primary result of our study was that IL-8 emerged as an undoubted marker for vestibulodynia. Interleukin 8 (IL-8), also known under a variety of alternative names such as chemotaxin, CXCL8, or NAP (neutrophil-activating factor), is a pro-inflammatory chemokine that mediates the chemotactic activity of various leukocytes. IL-8 has been suggested to play a significant role in a wide variety of diseases and pathologies including neurological, gastrointestinal, urological, metabolic, endocrine and wound healing problems, as well as arthritis, gingivitis, inflammation, inflammatory bowel disease, oncological diseases [21]. It is normally produced by different cell types in humans, including macrophages, fibroblasts, endothelial cells, keratinocytes, melanocytes, chondrocytes, epithelial

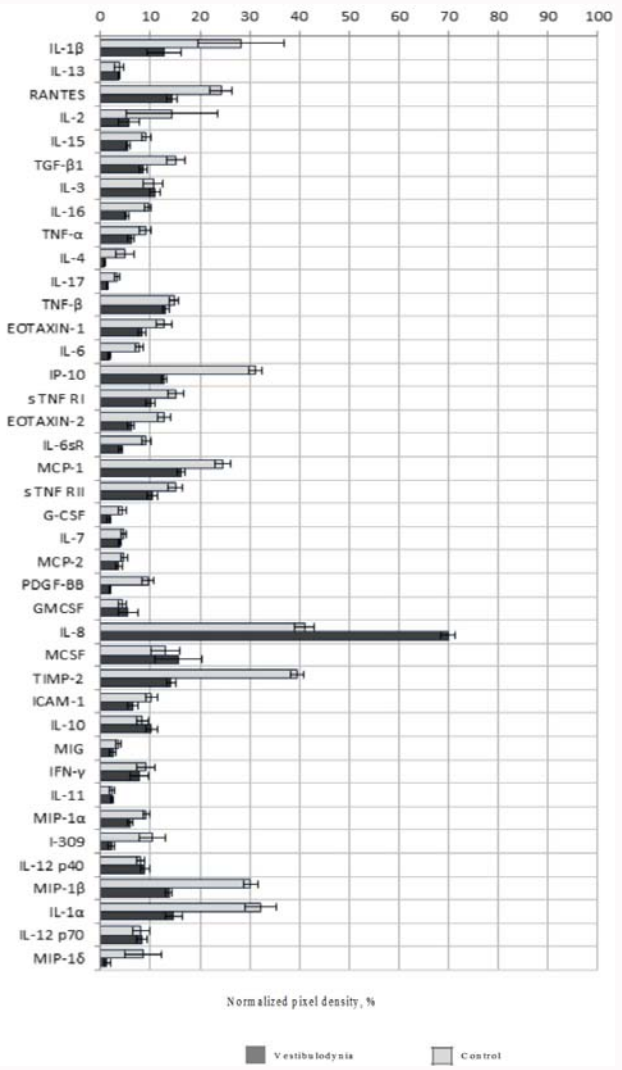

Figure 1a: Densitometric analysis of cytokines and chemokines in vestibulodynia patients vs. controls. Densitometric analysis was done as described in Materials and Methods. Shows I the levels of expression of up-regulated and down-regulated cytokines/chemokines in patients with vestibulodynia and control groups.

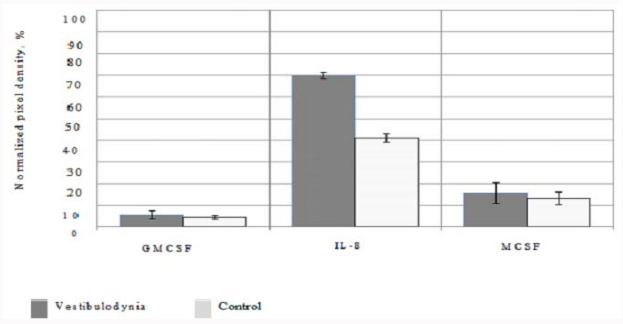

Figure 1b: Densitometric analysis of cytokines up-regulated in vestibulodynia *vs* control. Observed increase in cytokines expression in patients with vestibulodynia *vs*. control group. Asterisk indicates the cytokine (IL-8) with significantly (**p<0.001) increased expression.

cells, and mast cells [22]. IL-8 differs from all other cytokines in its ability to specifically activate neutrophil granulocytes [23,24]. After being released, IL-8 binds to its receptor, which can induce a variety of biological reactions, which include a transient increase of cytosolic calcium levels, the release of enzymes from granules, and enhanced expression and avidity of adhesion molecules [21]. Increased production of IL-8 is considered to contribute to a number of inflammatory disease, characterized by accumulation of activated neutrophils in the lesional areas [25]. IL-8 and other CXC chemokines preferentially act by inducing neutrophil trafficking across the vascular epithelium, while CC chemokines as MCP-1, MCP-3, RANTES, MIP-α, and MIP-1β (most of which were down regulated in our experiments), act not on neutrophils, but on monocytes, T-lymphocytes, and on basophil and eosinophil granulocytes [23]. The synthesis of IL-8 is not constitutive, but can be stimulated by some cytokines (e.g., IL-1 and TNF-α), and other factors, such as phytohemagglutinins, concanavalin A, double-stranded RNA, phorbol esters, sodium urate crystals, viruses, and bacterial lipopolysacharides (LPS) [21]. In addition, IL-8 secretion can be triggered by oxidant stress mediators. Higher IL-8 levels increase recruitment of inflammatory cells, which induce further increases in oxidant stress mediators, making IL-8 a key parameter in localized inflammation [21] Overall, IL-8 has been suggested as a biomarker for different diseases and pathological stages. [21,22,24,26-30]. Increased levels of IL-8 in primary culture of vestibular fibroblasts from vulvar vestibulitis patients, undergoing surgery of the lower genital tract, were shown both at baseline and after stimulation with Candida albicans *in vitro* [18]. IL-8 is also involved in mediating pain and in pain pathogenesis [21,30-33]. Pain can be related to both CNS disorders and peripheral nerve damage including chronic pain. The role of IL-8 In pelvic pain (PP)/ chronic pelvic pain (CPP) was described in a study on IL-8 as a biomarker of inflammation in benign prostatic hyperplasia (BPH) and chronic pelvic pain syndrome (CPPS) [30]. In chronic pain (CP) and CPPS research on whether levels of IL-1β, IL-2, IL-6, IL-8 and IL-10 were elevated in seminal plasma, only IL-8 correlated with symptoms in patients with CP/CPP [30]. Significant elevation of only IL-8 was detected in all patients with benign prostatic hyperplasia(BPH) and IL-8 was shown to be a reliable biomarker of inflammation in BPH and a predictive marker in CP/CPPS, and BPH [26,28,30]. It was also shown that the levels of IL-8 together with COX-2 were helpful in determining whether BPH was complicated by histological prostate inflammation [28]. Slone et al. [33] reported that IL-8 and serotonin share the same receptor, CXCR2, which is a possible explanation of IL-8's role in pain and inflammation [34]. Inflammatory mediators such as serotonin are pain-related as they excite and sensitize nociceptive neurons [35]. It has been shown that in the vestibular tissue of women with vestibulodynia, the number of cells expressing both the inflammatory mediator serotonin and CXCR2 are upregulated [33], which is important for pain perception [32,33]. Mast cells can also be part of the mechanism by which IL-8 is involved in inflammation and pain. The interaction between the nervous system and the immune system plays an important role in pain processing [13]. Mast cells are emerging players in physiological and pathological pain pathways [36].Mast cells induce nociceptor activation through the release of chemical mediators and thus can participate in signaling in neuro-immune synapses [37]. Mast cells are frequently found in close proximity to nociceptive neurons and therefore can participate in signaling in neuro-immune synapses [36]. The fact that mast cells residing in close proximity to unmyelinated nerve fibers is particularly important for understanding the pain conditions where mast cell-nerve associations have been documented, such as vulvodynia [38,39] and inflammatory bowel syndrome [40]. Mast cells induce nociceptor

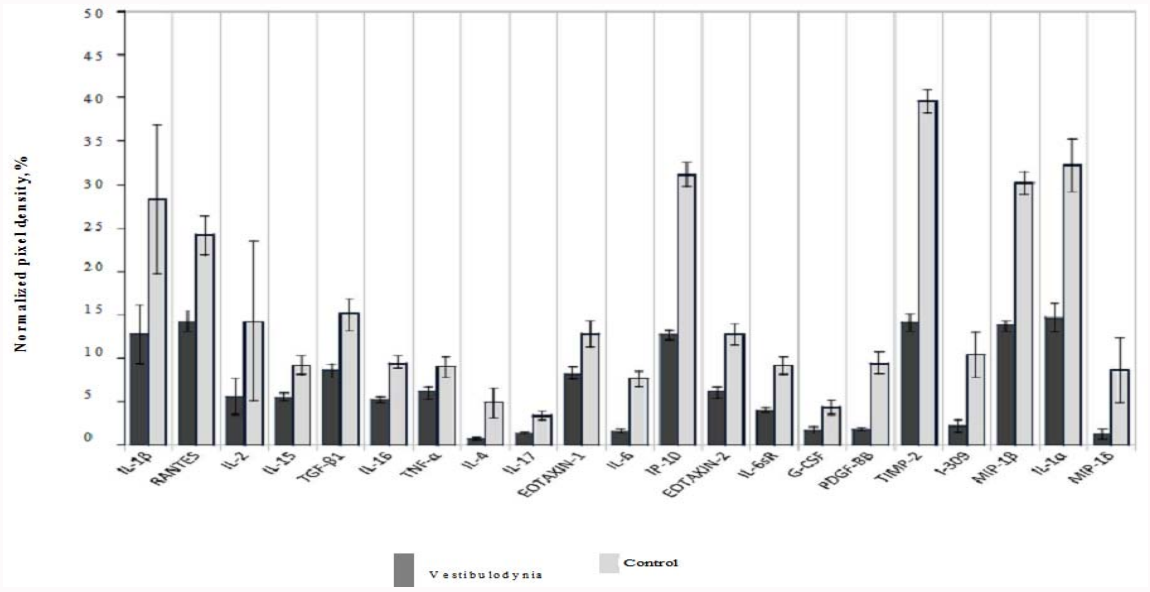

Figure 1c: Densitometric analysis of cytokines /chemokines down-regulated in vestibulodynia *vs*. controls. Observed significant (**p<0.005) decrease in levels of down-regulated cytokines/chemokines in vestibulodynia patients *vs*. controls.

activation through the release of chemical mediators during degranulation and can be activated by mediators released from nociceptors upon injury [13]. Active sensitization and challenge leads to pain that are dependent on mast cell degranulation. The injection of cytokines IL-6, TNFα, IL-1β, and IL-8 secreted by mast cells have been shown to cause hyperalgesia [41]. It has been reported that increased number of total mast cells, degranulated (e.g., IL-8 secreting) mast cells, and increased epithelial innervation were detected in vestibular biopsies of 40 women with vulvodynia [11]. There were also findings of increased mast cell numbers and innervation in tender *vs.* non-tender vestibular sites in 10 patients with primary provoked vulvodynia [13]. In addition, an increased risk of developing vulvodynia through potentially mast cell-dependent mechanisms was described by Harlow et al. [42], Bornstein et al. [11] have shown that the presence of 8 or more mast cells in a 10 x 10 microscopic field can be used as diagnostic criteria in localized vulvodynia (vulvar vestibulitis) [38]. The second histopathological criteria shows that total calculated area of nerve fibers in the area of vestibulodynia is ten times higher than expected [38]. Research has shown that direct contact between mast cells and T-lymphocytes leads to mast cell degranulation [43,44], followed by the release of IL-8 from mast cells. The IL-8 then stimulates neutrophil chemotaxis to the site of inflammation [45]. Mast cells may play a role in pain processing through a direct interaction with the nervous system. As was mentioned above, mast cell degranulation leads to IL-8 secretion (as well as some other interleukin secretion). Several studies have described a connection between mast cell activation and clinical pain disorders [13,41,46,47]. MCSF, monocyte-chemoattractant factor, and GM-CFS, granulocyte-macrophage chemoattractant factor, which were also elevated in vestibulodynia patients in our study, are secreted by macrophages, T cells, mast cells, NK cells, endothelial cells and fibroblasts, may stimulate production of macrophages and dendritic cells. Research has shown that MCFS and GM-CSF may act together with IL-8 [48]. Also, in our study, IL-12p40 and IL-12p70 were slightly elevated in vulvodynia patients (Table 1 and Figure 1). IL-12 can be produced by dendritic cells, which are stimulated, for example, by vaginal microbes. Dendritic cells are a type of antigen-presenting cells, which induce a primary immune response in the inactive T- and B-lymphocytes and act as messengers between the innate and adaptive immune responses [49,50]. As mentioned before, IL-1 β, RANTES, IL-2, IL-15, TGF-β1, IL-16, TNF-α, IL-4, IL-17, IP-10,EOTAXIN-1 and -2, IL-6, IL-6sR, MCP-1, G-CFS, TIMP-2, PDGF-BB, I-309, MIP-1β, IL-1α, , MIP-1δ are significantly ($p<0.001$, or $p<0.005$) down-regulated in vestibulodynia patients as comparison to healthy volunteers in our study (Figure 1c). So far there does not exist a definite conclusion about the whole profile of cytokines and chemokines involved in vestibulodynia and their role in this condition [18,51]. While in our study, IL-8 was significantly increased in the group of vulvodynia patients (number 32, $p< 0.001$) versus the control group (26, $p<0.001$), cytokines MCSF, IL-10 and GMCFS were also increased, while a whole group of cytokines was significantly down-regulated (Table 1and Figure 1c). We can only hypothesize why those cytokines were down-regulated. RANTES cytokine, together with the related cytokines MIP-1α, MIP-1β might be down-regulated as a response to insufficient ovarian steroid hormones [51,52]. It was shown [16,53] that women with vulvar vestibulitis have an increased amount of IL-1 receptor antagonist, and this genotype is associated with chronic inflammation in a variety of autoimmune disorders . A deficiency in IFN-α is typical for women with vulvar vestibulitis [16]. There are examples when IL-8 was up-regulated and IL-6 was down-regulated in human cells [54]. The IL-2 family includes IL-4, IL-7, IL-9, IL-15, IL-21. IL-2 is part of body's natural response to microbial infection [55]. In vestibulodynia there are no signs of active infection, and this is why IL-2 may be down-regulated. IL-6 and IL-6r, being leading factors in acute infections, are not involved in immune response in vestibulodynia. Monocytes and granulocytes (e.g. neutrophils) may down-regulate IL-6r and IL-6 [16]. IL-4 induces Eotaxin production [54,56-59]. In our experiments IL-4 is downregulated, so are Eotaxin-1 and IL-2. IL-17 which acts synergistically with TNF and IL-1 is also down-regulated [60,61]. At present the etiology of vestibulodynia remains unknown. Foster et al [6]. Suggested that vestibulodynia is an inflammatory condition, which appears to be the result of non-classical inflammatory and cytokine-mediated pain syndrome [18]. In our experiments using

vaginal rinses from 33 vestibulodynia patients and 26 healthy volunteers IL-8 appeared to be the prominent marker of both inflammation and pain syndromes of vestibulodynia.

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