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Cytokine profiles of women with vulvodynia: Identification of a panel of pro-inflammatory molecular targets

Nunzia Zanotta[a], Giuseppina Campisciano[a], Federica Scrimin[a], Ura Blendi[a], Annalisa Marcuzzi[b], Ezio Vincenti[c], Sergio Crovella[a,b], Manola Comar[a,b,*]

[a] Institute for Maternal and Child Health – IRCCS Burlo Garofolo- Via Dell'Istria 65/1, Trieste, Italy
[b] Department of Medicine, Surgery and Health Sciences, University of Trieste, Piazzale Europa 1, Trieste, Italy
[c] Dolo Medical Center, Department of Anaesthesia and Intensive Care, Via Guolo 15, Dolo, Italy

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ABSTRACT

Objective: The vulvar pain syndrome (VPS) is a multifactorial disease severely influencing the lifestyle of affected women. Among possible etiological factors, local injury, peripheral and/or central sensitization of the nervous system, and a chronic inflammatory status have been positively associated with the development of VPS. The identification of a constitutive altered local inflammatory profile in VPS women may represent an important point in the characterization of patients' phenotype as a useful marker influencing the vulvar micro-environment. The aim of this study was to investigative the possible role of the local cytokines production in women with VPS in comparison to healthy women.
Study design: In this study were collected vaginal swabs from 57 healthy women (HC) who never suffered from VPS and from 30 patients diagnosed with vulvodynia (VPS) by at least 3 years and currently symptomatic. All patients included in this study showed the absence of Sexually Transmitted (STD) diseases and Reproductive Tract Infection. Real-time PCR was performed to assess the genomic sequences of ST pathogens. The Luminex Bio-Plex platform was used for the analysis of a panel of 48 immune factors.
Results: Eleven molecules, specifically involved in the pro-inflammatory pathway were significantly modulated in VPS patients in comparison to healthy women, suggesting a persistent inflammatory process.
Conclusions: Therefore, these inflammatory factors could be possible biological markers involved in this disease. Nevertheless, other studies are needed to consider this specific immune profile as a valid marker of the vulvodynia.

Introduction

The International Society for the Study of Vulvar Diseases defines vulvodynia as chronic pain or discomfort condition, without any clear etiology, involving the vulva lasting more than 3 months, [1]. A combination of biological and social factors seem to characterize the disease that affects up to 16% of women across all age groups and ethnicities regardless of educational and socioeconomic backgrounds [2,3]. Vulvodynia is differentiated into localized and generalized subtypes, although both these are probably extreme variations of one syndrome [1]. Clinically, the vulvar pain syndrome (VPS) is considered a complex gynecological disorder mainly characterized by different grade of pain, sexual dysfunction and psychological distress [2]. VPS can affect women during all period of their life including both adolescent and post-menopausal phases whit a high prevalence of the reported cases occurring between 20 and 50 years [4]. Despite the many efforts made in the past decades to understand the pathogenesis of VPS, no specific and efficient treatment has been developed; the majority of therapeutic efforts are just basically envisaged to limit patients' pain [5,6]. At present, available treatments consist of combined topical therapies, pelvic physiotherapy, and drugs for pain control as well as an antidepressant in the tentative of reducing VPS impact on patients' life [7]. Among possible etiological factors, positively associated with the development of VPS, local injury and peripheral and/or central sensitization of the nervous system, leading to an inflammatory status, have been recently recognized. Histological evidence of local changes to the vestibular skin in patients suffering from vulvodynia shows chronic inflammatory infiltration, the massive presence of mast cells, and increased nerve fibers, suggesting a neuropathic pain

* Corresponding author at: Institute for Maternal and Child Health – IRCCS Burlo Garofolo- Via Dell'Istria 65/1, Trieste, Italy.
E-mail address: manola.comar@burlo.trieste.it (M. Comar).

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component [8,9]. In addition, microbiological causes inducing a fibroblast-mediated pro-inflammatory response that contribute to pain induction in localized provoked vulvodynia has been observed in women with *Human papillomavirus (HPV)* and *Candida albicans* infections [10]. Although additionally co-morbid pain factors such as inflammatory bowel disease, fibromyalgia and endometriosis have been also indicated in VPS patients, the activation of pro-inflammatory factors seems to represent a crucial trigger of VPS [11]. The identification of a constitutive altered local inflammatory profile in VPS women may represent an important point in the characterization of patients' phenotype as a useful marker influencing the vulvar micro-environment. With the aim of investigating the possible role of the local cytokines production in VPS pathogenesis, we analyzed a panel of cytokines/chemokines in VPS patients in comparison to healthy women.

Materials and methods

Vaginal samples

After approval by the Independent Bioethics Committee of the Institute for Maternal and Child Health – IRCCS "Burlo Garofolo" (L-1055 C.I.B. 09/02/2010), and after written informed consent and in accordance with the Declaration of Helsinki, vaginal swabs were collected, in menopausal women without hormone replacement treatment and in fertile women between day 22 and 28 of the menstrual period, no contraceptives, no previous sexual intercourse for 5 days. In this study were enrolled 57 healthy women (HC) (mean age 45.2, range 25–67 y) who never suffered of VPS (and/or autoimmune diseases) and 30 patients (VPS patients) (mean age 48.3, range 26–69 y) diagnosed with vulvodynia (VPS) by at least 3 years and currently symptomatic. Both women suffering from VPS, as well as healthy subjected included in this study, have been selected to have to be HPV, *C. tracomathis* and *Chlamydia T.* negative at the time of sampling, as revealed by the Bioplex based analysis. From the analysis of the clinical history combined with the physical examination, and with clinical symptoms, all enrolled women were negative to *Lichen Sclerosus or Planus,* and other vulvar dermatological disorders. In addition, HC and VPS patients showed the absence of other Sexually Transmitted Diseases and Reproductive Tract Infection, including *HIV, Gonorrhea, Syphilis, HSV.* Patients did not have a history of recurrent specific genital infections or acute events identifiable as triggers. VPS patients have been classified as ISSVD [2] and the evaluation of pain was based on Numeric Rating Scale (NRS) [12]. (NRS-11) is an 11–point scale for patient self-reporting of pain, and the patients of this study have reported an evaluation in the ratio of 7–10, corresponding to severe pain in the last 3–6 months.

Vulvoscopy in patients was negative, so biopsies were not performed due to the risk of reinforcing the neurological memory of pain. VPS patients and healthy controls (HC) were further classified into two different groups, considering age and presence of physiological menopause, named as Pre-menopausal group and Post-menopausal group (Table 1) [13].

Table 1
Demographic informations of the study groups.

Subjects	Number	Mean Age	Past Pregnancies
Healthy Control (HC)	57	42.2	25
VPS patients	30	48.3	12
HC Pre-menopause	45	43.6	20
VPS Pre-menopause	18	46.1	9
HC Post-menopause	12	55.2	5
VPS Post-menopause	12	58.5	3

HC = healthy controls; VPS = patients with Vulvar pain syndrome.

Microbiological and virology analysis

C. trachomatis and *HPV* were simultaneously detected in vulvar mucosa swabs using the bead-based Luminex suspension array technology (Luminex Corporation, Austin, TX). To amplify *C. trachomatis* DNA, a gene encoding for a conserved plasmid protein was used, with 10 copies as the detection limit of the assay. *HPV genotyping* was performed using the type-specific E7 polymerase chain reaction bead-based multiplex assay (TS-E7-MPG, IARC, Lyon, France), able to identify 27 *HPV* types (High-Risk-HPV types: HPV16-18-31-33-35-39-45-51-52-56-58-59-66-68-73-cp108; Probable/possible high-risk *HPV* types *HPV*26-5 3-6 7-69-82; Low-Risk-*HPV* types *HPV*6-11-55-81-83-84) and the β-globin gene as internal positive control. Viral genomes detected by the assay ranged from 10 to 1000 copies [14,15]. Briefly, HPV genotypes were detected as the median fluorescence intensity (MFI) of at least 100 beads per bead set. The background value for each probe was the MFI value resulting from the hybridization mixture without the addition of the PCR product. The cut-off was computed by adding 5 MFI to 1.1 the median background value. An additional set of *HPV* types including LR-HPV40-42-43-44-54-61-70 was identified by Anyplex™ II HPV Detection assay (Seegene Inc., Arrow diagnostics, Italy) using the CFX96 ™ Real-time PCR System (Biorad, France) as indicated by the supplier. A human housekeeping gene was used as an endogenous internal control to ensure DNA purification, PCR reaction and specimen quality. Candida strains were cultured and identified by traditional method using chromogen agar plates (Agar Chrom ID Candida).

Since Herpes Simplex virus infection may be a cause of vulvar pain, and this infection may arise in the vulvar area without any clinical lesion, all enrolled patients were tested for detecting a current (bead-based Luminex suspension array technology) [16], and a previous (serological testing, HSV 1–2 ELISA, Roche Molecular Diagnostics) HSV infection. In this study, only the women resulted negative for HSV from both tests were selected.

Determination of cytokines release

Soluble concentrations of a custom panel of 48 cytokines and chemokines (including: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, Eotaxin, Basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, PDGF-BB, MIP-1β, RANTES, TNF-β, VEGF, IL-1α, IL-2Rα, IL-3, IL-12(p40), IL-16, IL-18, CTACK, GRO-α, HGF, IFN-α2, LIF, MCP-3, M-CSF, MIF, MIG, β-NGF, SCF, SCGF-β, SDF-1α, TNF-α, TRAIL was assessed in duplicate performed on vulvar swabs using a magnetic bead-based multiplex immunoassays (Bio-Plex®) (BIO-RAD Laboratories, Milano, Italy) following manufactures' instructions. In brief, 50 μl of the undiluted vaginal swab, this amount falls within the linear range of the assay, and standards were added in duplicate to a 96 multiwells plate containing analyte magnetic beads. After incubation for 30 min at room temperature, wells were then washed 3 times with Bio-Plex wash buffer, and 25 u l of the premixed detection antibodies were added to the wells. This was followed by incubation for 30 min. After washing, 50 u l of streptavidin-PE was added to the wells and the plate was incubated for 10 min with shaking. The concentrations of the cytokines were determined using the Bio-Plex array reader (Luminex, Austin, TX). The data were analyzed using Bio-Plex Manager software (v.5, Bio-Rad), and were expressed as Median Fluorescence Intensity (MFI) and concentration (pg/mL).

Statistical analysis

The software GraphPad Prism (v. 5) (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical data analysis. For homogeneity