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## Developing Proteomic and Cytokine Biomarkers for Vulvodynia

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Developing Proteomic and Cytokine Biomarkers for Vulvodynia

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

By

ASHVIN IYER

Bachelor of Pharmacy, Mumbai University, 2012

2015

Wright State University

WRIGHT STATE UNIVERSITY  
SCHOOL OF GRADUATE STUDIES

Date: June 23, 2015

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Ashvin Iyer ENTITLED "Developing Proteomic and Cytokine Biomarkers For Vulvodynia" BE ACCEPTED IN PARTIAL FUFILLMENT OF THE REQUIRMENTS FOR THE DEGREE OF Master of Science.

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## **ABSTRACT**

Iyer, Ashvin M.S., Department of Pharmacology and Toxicology, Wright State University, 2015. Developing Proteomic and Cytokine Biomarkers for Vulvodynia.

Vulvodynia is a chronic, heterogeneous, and multifactorial disease. This condition may affect up to 18 percent of the female population including Caucasians, African Americans, Africans and Hispanics particularly those sexually active at child bearing age. The etiology of this condition is complex and multifactorial and it is frequently accompanied by physical disabilities, psychological distress and sexual dysfunction. Clinically, vulvodynia can be generalized or localized and pain can be provoked or unprovoked. Patients may also describe vulvar paresthesias or dysesthesias that may last hours. The International Society for the Study of Vulvar Disease (ISSVD) recognizes vulvar pain related to a specific disorder (infectious, inflammatory, neoplastic or neurologic) and vulvodynia (pain due to nonspecific etiology). In the case of vulvodynia, even the basic biological processes involved in the condition are unclear and further investigation of these processes is vital for constructing a substantive knowledge base. While recent research activities have created a foundation and enhanced our knowledge base, our understanding of the etiology and pathology of this condition is largely incomplete. Reports suggest a strong link that vulvodynia initiates a strong inflammatory reaction that is mediated by cytokines. In our study, we measured the levels of 27 cytokines that may be involved in this inflammatory response in the vaginal milieu of vulvodynia patients. For our study, we received 15 vaginal swabs from patients

diagnosed with vulvodynia and 5 vaginal swabs from healthy patients with no signs and previous history of this disease. Also the application of 2D-Difference in Gel Electrophoresis to study the presence and expression of different proteins in the vaginal milieu of vulvodynia patients has shown promising results. Our results indicate a more than 10-fold increase in IL-1ra levels ( $p<0.03$ ), a 7-fold increase in IL-12 levels ( $p<0.04$ ), a 3-fold increase in PDGF-bb ( $p<0.04$ ) levels, a 9-fold increase in VEGF levels ( $p<0.02$ ) and a 2-fold decrease in IL-17 levels in vulvodynia patients compared to healthy controls. Our results indicate the presence of a strong inflammatory response involved in vulvodynia. Our proteomic studies indicate alterations in the normal proteomic levels in the vaginal milieu of vulvodynia patients compared to healthy controls.

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## **INTRODUCTION**

### **Background**

Vulvodynia is a common genital problem affecting the vulvar region in women. It has been reported to occur in nearly 8% of women (Edwards 2015). Patients with this disorder usually complain of vulvar discomfort which includes burning, and any combination of stinging, irritation, itching, pain, rawness and dyspareunia (Masheb et al. 2000). This disorder has been recently recognized and is reported to affect 200,000 women in the United States (Goetsch 1991). This condition has been observed in all age groups but mainly observed in women during their reproductive years. The development of the disease can be acute or attained gradually (Burning Vulvar Syndrome 1984). The symptoms associated with the condition can be constant or encountered intermittently (Marinoff and Turner 1992). Quality of pain can be localized to a particular region in the vulva or spread throughout the vulva and the pain can be sensed deep inside the vulva or superficially (Lynch 1986). Pain suffered by such patients is usually moderate to severe (Lynch 1986). Any actions that bring pressure to the vulva can aggravate the pain in this condition (Friedrich 1987). Simple tasks like intercourse, insertion of tampons and speculum, tight-clothing and activities like bicycling, horseback riding or even simple exercises can aggravate the pain (Friedrich 1987).

## **Subtypes of Vulvodynia**

Vulvodynia is seen as the most difficult challenge faced by medical practitioners dealing with vulvar disorders. Five subtypes of this condition have been reported (Table 1) (McKay 1988). The first subtype, vulvar dermatoses, includes diagnosis of lichen sclerosis, lichen planus, chronic dermatitis and eczema (Masheb et al. 2000). Cyclic candidiasis/vulvitis is the second subtype and includes diagnosis of repeated candida infections. This stage can be confirmed by running a swab test for cultures which usually occur during the menstrual cycle (Masheb et al. 2000). The third subtype, vulvar papillomatovirus, includes the presence of a small papillae around the vestibule. Usually this subtype has been observed to be present with human papillomatovirus (Masheb et al. 2000). The fourth subtype, essential vulvodynia, includes dysesthesia, caused by nerves which bring discomfort similar to that seen in postherpatic neuralgia (Masheb et al. 2000). The fifth subtype, vulvar vestibulitis syndrome, includes discomfort and increased sensitivity in the vulvar vestibule (Masheb et al. 2000). This subtype may have an absence of physical findings with the exception of erythema (Masheb et al. 2000). This classification is one of many and has not been used by many researchers in the field. There is a lot of overlapping among the various classifications and a more accurate and detailed classification is required (Masheb et al. 2000).

#### Differential diagnoses of vulvodynia

| Subtypes                         | Symptoms/definition  |
|----------------------------------|--|
| <b>Vulvodynia</b>                |  |
| Vulvar dermatoses                | Stinging, itching or burning associated with dermatoses (e.g. irritant or contact dermatitis; lichen sclerosus or lichen planus; psoriasis)          |
| Cyclic candidiasis/vulvitis      | Itching, burning or swelling; symptoms may flare with menses and/or intercourse; history of positive culture for candida                             |
| Vulvar papillomatovirus          | Irritated mucosa; small papillae found around vulvar vestibule; HPV may be present   |
| Essential/dysesthetic vulvodynia | Constant burning; pain not related to touch; usually found in postmenopausal women   |
| Vulvar vestibulitis              | Dyspareunia; pain related to touch/pressure  |
| <b>Dyspareunia</b>               | Pain with intercourse that may be superficial, deep or both; may occur during or after intercourse; and may or may not be due to a medical condition |
| <b>Vaginismus</b>                | Recurrent or persistent involuntary muscle spasm which interferes with sexual intercourse  |
| <b>Pelvic pain</b>               |  |
| Physiologic                      | Pain associated with menstruation  |
| Organic                          | Pain associated with reproductive organs or pregnancy  |

Table 1: Diagnosis of Vulvodynia (Masheb et al. 2000)

### Significance

International Society for the Study of Vulvar disease (ISSVD), in 1985 recognized vulvodynia syndrome (Itza et al. 2012). This condition is a common reason for consultation among women. Patients first consult with their family physicians, then

they seek help from gynecologists, dermatologists, neurologists or psychiatrists (Itza et al. 2012). Patients end up visiting many doctors in search of a treatment making this condition a very frustrating one. Associated with the disorder are anxiety, severe depression and sexual dysfunction that further complicates the disease (Green and Hetherton 2005).

There are several etiopathogenic theories for vulvodynia. Women mostly report vaginal candidiasis as a condition suffered in their past (Itza et al. 2012). Reports suggested an allergic contact sensitization of candida (Ramirez De Knott et al. 2005). Studies also claimed that the disorder may be due to calcium oxalate crystals in the urine which may cause the vulvar burning. But this hypothesis was later rejected (Harlow et al. 2008).

### **Demographics**

Vulvodynia affects 200,000 women according to the New York Times. Reports suggest that vulvodynia is found in Caucasians (Friedrich 1987; Furlonge et al. 1991; Mann et al. 1992; McCormack 1990; Peckham et al. 1986), African Americans (Mann et al. 1992) and Hispanics (Friedrich 1987; Furlonge et al. 1991; Mann et al. 1992) but predominant in Caucasians. More than 65% of women who are suffering from this disorder range between 20 to 40 years of age (Friedrich 1987). Vulvodynia has had a significant burden to society, the health care industry, the patient suffering from this disorder and her family (Sadownik 2014). The annual economic burden of vulvodynia has been reported to be \$31-\$72 billion in the USA (Xie et al. 2012). This figure represents all costs associated with direct and indirect health care costs (Sadownik 2014). Many patients have to take leave from work which adds to the indirect costs (Sadownik 2014). Another problem is that most women do not seek medical attention when they are

affected by the disease. They usually treat themselves by applying some creams and lotions which tends to make the condition more severe. Patients with vulvodynia suffer from depression (Schover, Youngs, Cannata 1992) and report to the reduction in the quality of life (Arnold et al. 2006). Many women suffer with frustration (Nunns and Mandal 1997), anxiety (Nunns and Mandal 1997), stress (Ehrstrom et al. 2009) and severe depression (Schover, Youngs, Cannata 1992). This leads to decrease in sexual activity and level of satisfaction achieved (Gates and Galask 2001; Sadownik 2000). The pain associated with vulvodynia has a negative influence on the patient's interpersonal relationship (Sadownik 2014).

### **Anatomy of Vulva**

The vulva is the female genital organ that is present externally. Oftentimes it is confused with vagina. It basically comprises the labia minora, labia majora, clitoris, mons pubis and the vestibule (Puppo 2011).

The vagina represents an internal genital organ. The urethrovaginal septum separates the vagina from urethra. The vagina has no secretory glands present (Gray, Williams and Bannister 1995; Testut and Latarjet 1972; Dickinson 1949). The Bartholin's gland is located at the ends of the vaginal wall (Gray, Williams and Bannister 1995; Testut and Latarjet 1972; Dickinson 1949). These glands have been reported to secrete viscous secretions just before attaining peak sexual pleasure (Masters and Johnson 1966). Endocrine cells have been found in the Bartholin's gland that produce serotonin, calcitonin, bombesin and katacalcin (Fetissof et al. 1989). Many mucus glands open in to the vestibule called minor vestibular glands.

The mons pubis is largely made up of adipose tissue. It's a skin layer composed of sebaceous glands and sweat glands and is almost entirely covered with pubic hair (Sokol A and Shveiky D, 2008).

Labia Majora consists of two longitudinal folds that extends inferiorly and posteriorly from the mons pubis. It consists of several glands and is covered with hair (Gray, Williams and Bannister 1995; Testut and Latarjet 1972).

The labia minora represent two small cutaneous fold which range from 3-4 cm in length (Puppo 2011). The two folds are very closely located and the labia minora in general are unrecognizable (Puppo 2011). The upper parts converge together and form the prepuce. The posterior parts converge and form frenulum (Gray, Williams and Bannister 1995; Testut and Latarjet 1972; Dickinson 1949). Labia minora contain thick erectile connective tissue that contain many small vascular networks for blood flow. During sexual stimulation, this region becomes engorged with blood causing it to increase in thickness up to three times (Puppo 2011; Shafik, Shafik, Ahmed 2004; Yang et al. 2006).

The clitoris is the primary female sex organ. It is extensively supplied with nerve endings. The crura, body and glans make up the clitoris (Gray, Williams and Bannister 1995; Testut and Latarjet 1972; Dickinson 1949). They basically are made of spongy tissues which become rigid during coitus (Gray, Williams and Bannister 1995; Masters and Johnson 1966; Testut and Latarjet 1972; Dickinson 1949; Yang et al. 2006).

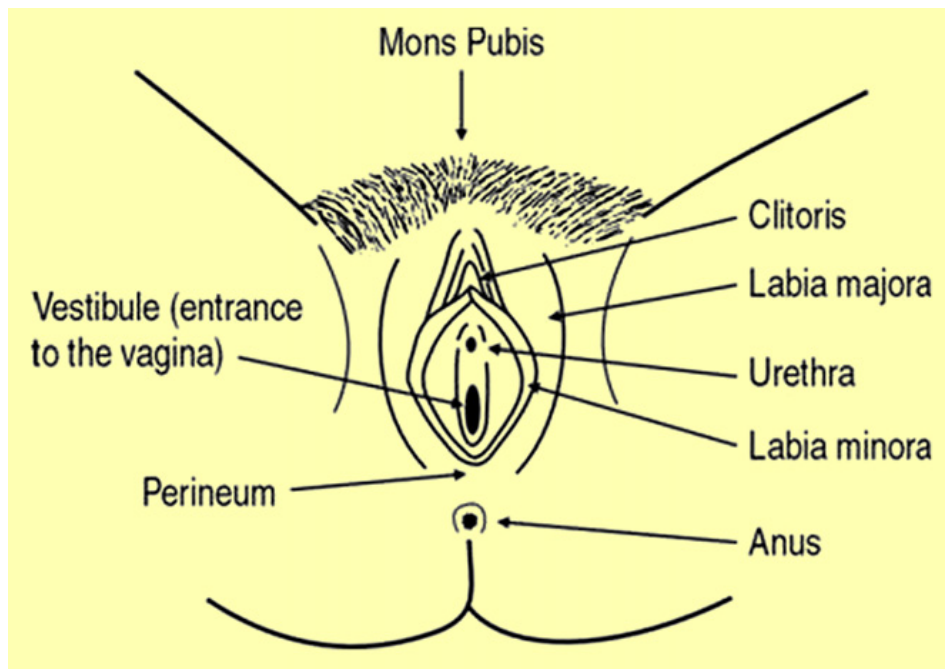


Figure 1: Vulval Anatomy (courtesy of the Vulval Pain Society)

## **Management of Vulvodynia**

### **General Vulvar Care**

First step is to eradicate the contact of all potential irritants which may take the form of creams (O'Hare and Sherertz 2000). Other commonly applied products like fragrances, sanitary products, cleansing gels and douches and products containing alcohol should be prohibited (O'Hare and Sherertz 2000; Segal 2008). Application of ice or cold gel packs to the vulva has been found to be helpful in alleviating the pain (Segal 2008). Regular cleaning of vulva with mild soap or an emollient with no preservatives can be applied to bring in moisture to the skin and improve its protective function (Haefner et al. 2005) . The above-mentioned remedies have not been used successfully in the treatment

of moderate to severe conditions of vulvodynia (Davis and Hutchison 1999), suggesting only prevention of symptoms.

### **Topical Therapies**

Application of 5% lidocaine ointment every night for a period of 6 months resulted in improvement in the ability of women to have intercourse (Zolnoun, Hartmann, Steege 2003). It is thought to inhibit the feedback transmission of pain (Zolnoun, Hartmann, Steege 2003). Lidocaine 2% or 5% in the form of jelly or an ointment can be applied prior to sexual intercourse (Segal 2008).

Clinical trials have reported capsaicin to have a protective effect on the inflammatory and neurologic disorders that are commonly seen in patients with vulvodynia (Fugh-Berman 2003). Capsaicin is reported to cause a desensitization reaction which is carried out by neuropeptides, involving substance P (Steinberg et al. 2005). Other agents include nitroglycerin cream which seems to provide temporary relief from dyspareunia (Walsh et al. 2002) and amitriptyline 2% with baclofen cream 2% (Nyirjesy 2009).

### **Oral Therapies**

Tricyclic antidepressants are a popular choice for the treatment of neuropathic pain associated with vulvodynia (Segal 2008). Amitriptyline is the first line treatment initiated on low doses and slowly increasing the dose up to 150 mg per week (Haefner et al. 2005). Common side effects associated with its regimen include sedation, weakness, dizziness, lethargy, dry mouth and palpitations (Segal 2008). Another class of drugs, the selective serotonin reuptake inhibitors (SSRIs) have been included in the regimen for treatment of vulvodynia (Edwards 2003). Patients who do not respond to tricyclic



antidepressant are switched over to gabapentin treatment (Segal 2008). Gabapentin is a well-known anticonvulsant and has been effective in controlling neuropathic pain (Segal 2008). Several weeks of dosing are required to reach maximum efficacy. Gabapentin has fewer side effects compared to the tricyclic antidepressants (Segal 2008). Carbamazepine is another anticonvulsant drug that can be used in case of patients not responding to gabapentin (Haefner et al. 2005).

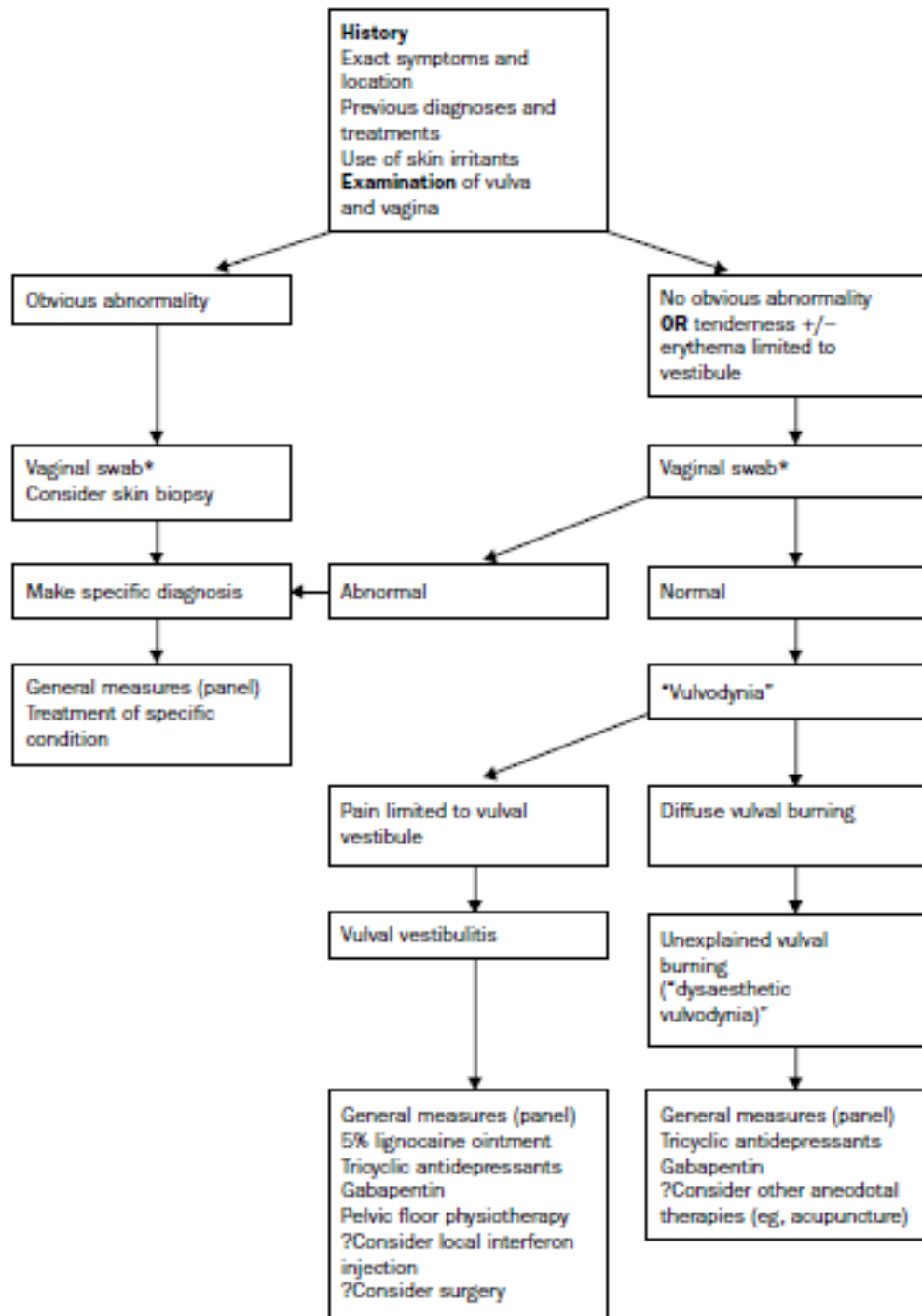
### **Physical Therapy**

Changes brought to the pelvic floor muscles, which may accompany pelvic floor tone, instability of these muscles resulting in loss of muscular control are usually associated with chronic vulvodynia (Sadownik 2014). Physical therapy educates patients on their pelvic floor muscles, increasing their potential to gain control of these muscles in order to contract, and relax completely, and address muscle spasms (Sadownik 2014). Three simple exercises are currently employed by gynecologists in order to counsel patients with vulvar pain. Kegel's maneuver, reverse Kegel's maneuver and superficial perineal massage (Sadownik 2014). Patients are encouraged to follow these exercises up to 10 sets in a day (Sadownik 2014).

### **Surgical Options**

Surgery is usually reserved for patients who are non-responsive to conventional treatments. Surgery can be divided into local excision, vestibuloplasty and total vestibulectomy (Masheb et al. 2000). Based on the extent of vestibular involvement, the excision should be tailored to the individual patients (Davis and Hutchison 1999). It is important that patients first be subjected to a cotton swab test which helps in locating areas of the vestibule experiencing pain and which may need to be surgically removed

(Masheb et al. 2000). Local excision involves locating the problem areas and removing the affected tissue in an elliptic manner. (Haefner et al. 2005). Vestibuloplasty involves excision of the anterior, posterior or lateral vestibule without the involvement of the vagina (Haefner 2000). Vestibulectomy involves cutting of the complete vestibule from the urethra including the hymen (Masheb et al. 2000). It is the most popular choice of surgery for women with provoked vulvodynia (Masheb et al. 2000). Success rate range from 60% to 94% after this surgery (Davis and Hutchison 1999). The drawbacks of this surgery are the cost and risks associated with the removal of glands that function to lubricate. Scarring of tissues during surgery is another drawback that is seen with this type of surgery (Masheb et al. 2000).



### Management of chronic vulval burning/pain

\*Vaginal swab should be routinely done for microscopy (yeasts, leucocytes, bacterial flora, maturity of epithelial cells) and culture.

Figure 2: Management of Chronic Vulval burning (Helen E. Lotery et al, 2004)

## **Role of Cytokines in Inflammation**

Cytokines are small glycoproteins produced by various cells but mostly helper T cells and macrophages (Zhang and An 2007). They are associated with the regulation of immunity, inflammation and hematopoiesis (Khan 2008). These mediators are involved in promoting the proliferation and differentiation of hematopoietic cells and in regulation and determining the nature of the immune response (Thèze 1999). Each cytokine has several effects on different types of cells. Interactions exist between different cytokines and can be synergistic or inhibitory (Thèze 1999). Cytokines induce their actions by binding to specific receptors on the cell surface. The target cells respond by new mRNA and protein synthesis that results in the specific biological response (Khan 2008). When a peripheral nerve injury takes place, macrophages and Schwann cells accumulate at the injury site and secrete cytokines and other mediators for repair (Zhang and An 2007).

Pro-inflammatory cytokines participate in the up-regulation of inflammation (Zhang and An 2007). The main cytokines of this category are  $\text{TNF}\alpha$ , IL-1, IL-6, IL-8 and macrophage inflammatory protein-1 $\alpha$  (Jaffer, Wade, Gourlay 2010). The presence of these cytokines have been reported to be involved in many injuries (Halter et al. 2005; Mokart et al. 2002). Anti-inflammatory cytokines suppress the actions of pro-inflammatory cytokines. IL-1ra, IL-10 and IL-13 constitute the major anti-inflammatory cytokines (Jaffer, Wade, Gourlay 2010; Zhang and An 2007). Among them, IL-10 is the most potent and inhibits the expressions of  $\text{TNF-}\alpha$ , IL-6, and IL-1 (Zhang and An 2007). IL-10 has the property of inducing anti-inflammatory cytokines and inhibiting pro-inflammatory cytokines (Zhang and An 2007). A study reported that low blood levels of

anti-inflammatory cytokines IL-10 and IL-4 was associated with patients with chronic pain (Uceyler et al. 2006).

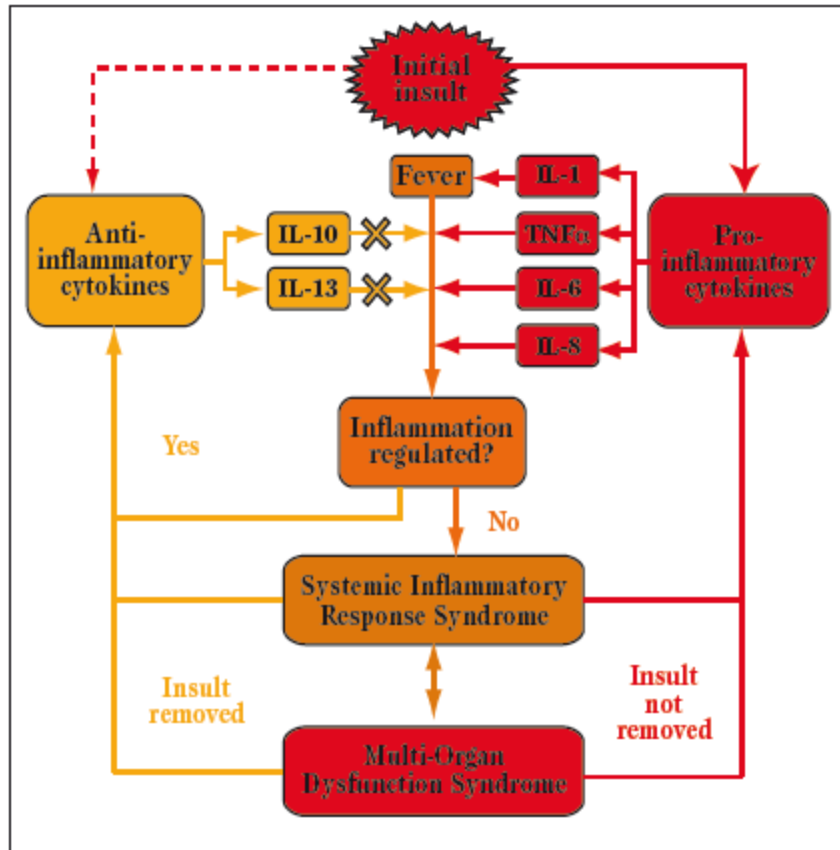


Figure 3: Response to insult (Jaffer, Wade, Gourlay 2010)

Seckin-Alac et al. (2014) compared the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-1 receptor in women with vulvar vestibulitis syndrome with control patients. They found a significant difference in TNF- $\alpha$ . Positive staining has been found in 40% of the VVS samples and in 7.7% of the samples from the control group. Their intention was to suggest that the local inflammation may be mediated by cytokines in VVS patients.

Clinical and research studies have reported vulvodynia patients to have low serological levels of interleukin-1 receptor antagonist which might suggest such patients to be exposed to a greater risk of a pro-inflammatory immune response as a result of their inability to terminate IL-1 production during an inflammatory reaction (Gerber et al. 2002). Studies have shown women with vulvodynia to have a higher frequency of alleles in IL-1B and IL-1 receptor polymorphic genes (Gerber et al. 2002; Jeremias, Ledger, Witkin 2000).

Foster et al. (2007) conducted a study where they challenged VVS patients with candida albicans (yeast) and alpha-melanocyte-stimulating hormone in-vitro. They found that coincubation with  $\alpha$ -MSH plus yeast resulted in significant 3-fold increase in IL-6 and more than 40-fold increase in IL-8 in VVS patients and controls. Vestibular fibroblasts from VVS patients produced high levels of IL-1 $\beta$ , IL-6 and IL-8 at baseline and following yeast-alone challenge.

### **Introduction to 2D-Difference Gel Electrophoresis**

2D Fluorescence Difference Gel Electrophoresis (2D-DIGE) is an advanced method for labeling of proteins that make use of CyDye Fluors. They utilize the principle of 2-Dimensional gel electrophoresis. This technology simplifies the process of detecting and identifying proteins using the 2-D electrophoresis and allows up to three different proteins samples to be analyzed on the same gel.

The three different protein samples are labelled with different CyDyes. Usually the control samples are labelled with Cy5, the treated or diseased samples are labelled with Cy3 and an internal standard consisting of a mix of all the control and treated samples are labelled with Cy2. After labelling of samples, they are pooled together and

are subjected to the first dimensional separation based on the isoelectric points and subsequent second dimensional separation based on molecular weight using the SDS PAGE gels.

The advantage of this technique is that it allows multiplexing different samples to the same first and second dimensional separation that helps limit experimental variation. The individual samples are then visualized independently using individual excitation and emission wavelengths for each CyDye. Another advantage of this technique is that by running multiple samples on a single gel, the number of gels required to produce the same data is reduced. The internal standard represents all proteins from every sample that is present on all the gels. This increases the confidence of matching between gels and allows the generation of accurate spot statistics between gels.

The CyDye Fluors are dyes that have a reactive NHS ester group. In the presence of a protein sample, they covalently bind through an amide linkage to the epsilon amino group of lysine of the protein sample. Cy3, Cy5 and Cy2 dyes are matched for size and charge so that the three labelled protein samples are subjected to the same 2-D gel electrophoresis separation.

#### Application of 2D-DIGE for Biomarker discovery

Grubbs et al., (2015) successfully labelled porcine serum using the 2D-DIGE methodology and proved that the serum can be used for biomarker discovery. Yu, Rustgi, and Blair (2005) used this approach to identify biomarkers for pancreatic cancer. They utilized serum samples from patients with pancreatic cancer and depleted the serum of the abundantly found proteins and successfully labelled with CyDyes.

Huang et al. (2006) also utilized this technique and identified an increased expression of transferrin in the serum of patients with breast cancer. Dutta et al. (2015) investigated serum proteins of patients with endometriosis using 2D-DIGE and compared the results with healthy control patients using MALDI-TOF/TOF MS. This analysis resulted in 25 significant different proteins. They concluded that haptoglobin (HP), Ig kappa chain C region (IGKC), alpha-1B-glycoprotein (A1BG) can be considered effective serum protein diagnostic markers for different stages of endometriosis.

In a study using interleukin 10 knockout mice, circulating proteins from serum samples were identified from different age groups of mice to detect biomarkers for inflammatory bowel disease. Fifteen different proteins were identified by 2D-DIGE and MALDI-TOF/TOF mass spectrometry in comparison with non-inflamed mice. The identified proteins were confirmed by ELISA and western blots (Viennois et al. 2015).

Huang et al. (2014) reported transthyretin, retinol binding protein 4, haptoglobin, clusterin, serum amyloid A protein, apolipoprotein A-I, apolipoprotein C-III and apolipoprotein C-II to be expressed in significantly different levels in patients with Occupational medicamentosa-like dermatitis induced by trichloroethylene compared to healthy controls. They identified these proteins by 2D-DIGE and MALDI-TOF MS/MS and confirmed them by western blots.

2D-DIGE was successfully used to identify seventeen different proteins among patients with normal but non-fertilizing sperm and patients with normal fertilization. Twelve proteins were identified by mass spectrometry and they found two proteins, laminin receptor LR67 and L-xylulose reductase which may influence gametes interaction (Frapsauce et al. 2014). Wu et al. (2014) identified glucose-regulated protein



78, glutathione s-transferase pi, apolipoprotein AI, alpha-1 antitrypsin and gastrokin-1 in gastric cancer tissues obtained from surgery by 2D-DIGE and confirmed them by western blots.

### **Preliminary Studies**

My team is currently working in 3 areas

- 1) A double-blinded study was done where vaginal samples were assessed for bacterial flora, cytokine and proteomic analysis. Samples were obtained from patients between days 22 and 28 of their menstrual period, with no previous sexual intercourse for 3 days and on no medications or contraceptives. This pool of 17 patients was divided into three groups; i) symptomatic patients with vulvodynia, ii) patients with vulvodynia in remission and iii) patients with no history of vulvodynia which were used as controls for the study. The study showed that all 17 patients had a negative culture for fungi and were negative by PCR for *Atoprobium*, *Megasphaera* and *Leptorichia*.

Preliminary data obtained from Microbiological studies are very promising. Patient samples used as controls showed the presence of *L. Crispatus* which was not found to be present in patients affected with symptomatic vulvodynia or in remission. Symptomatic patients and patients in remission showed the presence of *L. Gassery*. *L. Iners* was found to be present in both controls and vulvodynia samples and was found not to play any role in the disease. These results suggest the presence of an alteration in the vaginal flora in patients with vulvodynia. From our analysis, we found differences in *Lactobacillus* species. As the study was done in small sample population, it is difficult to say that these results would be consistent to the major

population with this disease. Reports suggest that all *L. Crispatus* and 71% of *L.*

*Gassery* produce hydrogen peroxide. *L. Crispatus* promotes in pregnant patients the stability of the normal microflora and *L. Gassery* and *L Iners* are more conducive to the occurrence of abnormal vaginal flora (Verstraelen et al. 2009).

- 2) Cytokine analysis was performed on 13 samples with 6 controls and 7 patients with vulvodynia. We utilized the Bio-plex bead based system which assayed 27 different cytokines in these patient samples. Patients with vulvodynia showed an increase of nearly 35 times interleukin-17 (IL-17) compared to control patients. Also these patients showed a 7 fold decrease in macrophage inflammatory protein 1 beta (Mip-1b) and a 3-fold decrease in Interleukin-12 (IL-12) compared to control patients. This preliminary results suggest the involvement of an immunological response involving candida in patients with vulvodynia.

Cytokine analysis of vulvosecretions in vulvodynia.

| Cytokine     | Control (ng/ml) | Vulvodynia (ng/ml) | Change    |
|--------------|-----------------|--------------------|-----------|
| IL-1ra       | OOR             | OOR                | -         |
| IL-1b        | OOR             | OOR                | -         |
| IL-2         | OOR             | OOR                | -         |
| IL-4         | 1.12 ± 0.01     | 1.87 ± 0.24        | Increase  |
| IL-6         | 18.80 ± 1.08    | 18.85 ± 1.05       | None      |
| IL-7         | 6.31 ± 0.73     | 12.54 ± 2.15       | Increase  |
| IL-8         | OOR             | OOR                | -         |
| IL-9         | 10.71 ± 2.06    | 43.60 ± 10.25      | Increase  |
| IL-10        | 11.44 ± 1.37    | 7.26 ± 1.19        | Decrease  |
| IL-12        | 181.49 ± 8.39   | 52.12 ± 7.67       | Decrease  |
| IL-13        | 23.93 ± 2.72    | 26.78 ± 5.16       | None      |
| IL-15        | OOR             | OOR                | -         |
| IL-17        | 1.32 ± 0.07     | 44.92 ± 3.02       | Increase  |
| Eotaxin      | OOR             | OOR                | -         |
| FGF basic    | OOR             | OOR                | -         |
| G-CSF        | 27.13 ± ND      | 36.71 ± 5.035      | None      |
| GM-CSF       | 2947.25 ± 29    | 3396.60 ± 241      | Increase? |
| IFN-γ        | 531.56 ± 39.41  | 1518.25 ± 320      | Increase  |
| IP-10        | 16850.00 ± 0    | 5912.52 ± 326.8    | Decrease? |
| MCP-1 (MCAF) | 93.11 ± 0       | 65.63 ± 2.59       | Decrease? |
| MIP-1a       | 3.66 ± 0        | 41.99 ± 9.21       | Increase? |
| PDGF-b       | 74.02 ± 15      | 235.47 ± 47.0      | Increase  |
| Mip-1b       | 173.32 ± 2.73   | 24.00 ± 2.64       | Decrease  |
| RANTES       | 74.78 ± 0.78    | 158.68 ± 33.64     | Increase  |
| TNF-a        | OOR             | OOR                | -         |
| VEGF         | OOR             | OOR                | -         |

OOR, out of range (usually below threshold).

ND, not enough samples in range to determine.

Data represents mean ± SEM.

Table 2: Cytokine analysis of vulvosecretions in vulvodynia

- 3) The data regarding proteomic analysis via MALDI-TOF mass spectrometry is not significant although it suggests a tendency to demonstrate a difference between patient with vulvodynia and controls. Our preliminary sample was small and some samples were not adequate for this type of analysis, lessening the quality of our data.

A larger population of samples will ensure adequate sample numbers to provide meaningful data.

## **HYPOTHESIS**

Vulvodynia has been found to be a highly prevalent, chronic and multifactorial disease. Our preliminary studies have created a strong scientific base by enhancing our knowledge about this condition. Therefore a strong understanding of the etiology and pathology of this condition is vital to draw further conclusions.

I hypothesize that:

- Vulvodynia may involve an alteration in the balance of cytokines and proteins expressed in the vaginal milieu that maintains an inflammatory response.

My hypotheses will be tested by using a multidisciplinary approach including: proteomics and cytokines evaluation of the vaginal milieu. Samples will be obtained from patients who are suffering from vulvodynia and with patients with no history of vulvodynia which will be used as controls for the study.

I will evaluate the following:

- Types of cytokines present in patients with this condition
- The presence of any atypical proteins in the vaginal milieu of patients with this condition.

## **Specific Aims**

**Specific Aim 1:** To Identify and characterize cytokines present in patients with this condition

Inflammatory reactions have been previously described that strongly suggest a correlation with the change in bacterial infections. The goal of this aim is to determine on a more extensive level, changes in cytokines that may be involved in this inflammatory reaction. Previous studies have focused only on specific cytokines. In contrast, we propose to use a multiplexed bead-based system to simultaneously examine 27 cytokines at from each sample. Preliminary studies indicate that our method of collection, i.e., a swab used to collect the secretions dipped into a collection tube containing PBS and media, is adequate to analyze cytokines in this manner. Protein measurements were generally in the upper range of the standard curve indicating plenty of protein was acquired. The goal is to establish evidence of cytokines to fulfill the RFA request to “Identify and validate biomarkers that may be used to diagnose disease, predict disease development or progression, and stratify patient subsets or response to therapy, including biomarkers at the molecular, gene/protein and cell level.”

**Specific Aim 2:** The vaginal milieu respond to the insult by changes in proteins and/or abnormal protein production

To more fully understand the changes in the vaginal milieu, a more complete profile of the non-bacterial and non-cytokine proteins and other macromolecules should be obtained. Mass spectrometry is a commonly employed technique to achieve this as it is more sensitive and can provide specific protein/peptide identification via direct sequencing. The goal of this aim is develop a proteomic profile for normal versus vulvodynia patient secretions. Following this, protein/peptide sequencing will be done to attempt identification of unique proteins/peptides.

## **MATERIALS AND METHODS**

### **Strategy**

The protocol for all Specific Aims will include the following visits:

Exhaustive history and comprehensive assessment of environmental factors, topical vulvovaginal irritants and co morbid conditions acknowledged in the exclusion criteria. Additionally, psychosocial contributing factors, daily stressors, pain assessment other associated symptoms including previous sexual experiences and current sexual activity.

Detailed physical and pelvic examination samples will be obtained for proteomics, cytokines, vaginal lactobacillus and facultative anaerobic bacterial flora analysis from vaginal secretions and we will procure specimens for vaginal fungal cultures.

The above described protocol could be schematically divided in 4 parts:

- I. diagnosis-confirmation of vulvodynia, by history and physical examination: the diagnostic approach is currently utilized by most researchers including our team
- II. exclusion of co morbid conditions that could interfere with the protocol
- III. exclusion of fungal and facultative anaerobic bacteria infections
- IV. analyzing the role that proteomics and cytokines play in vulvodynia



## **Inclusion Criteria**

The patient group will consist of women ages 12 to 60 suspected or currently diagnosed with vulvodynia, or previously diagnosed and currently symptomatic. The control group was composed of asymptomatic and otherwise healthy women with no previous history of any chronic or recurrent vulvovaginal condition matched to the study group for age, race and parity. The control samples were obtained from patients who attend our collaborator's clinic for their regular check-ups. Patients were explained about the study and the ones ready to participate gave their informed consent and agreed to sign and follow the protocol.

## **Exclusion criteria**

Patients were excluded from the study under the following protocols:

- I. Aged under 12 and above 60 years old.
- II. Undergoing surgical treatment for vulvodynia or other vaginal complications or who are currently on therapy or in remission from vulvodynia.
- III. Diagnosed with medical conditions like cancer or a condition that is not under control like diabetes mellitus, hypertension, collagen disease, hemoglobinopathy, renal insufficiency and others.
- IV. Having Neurological or psychiatric complications including post-traumatic stress disorder and alcohol and/or drug addiction.
- V. Patients who have chronic pain conditions including fibromyalgia, levator ani syndrome, pelvic floor tension myalgia, interstitial cystitis, endometriosis and irritable bowel syndrome.
- VI. Pregnant patients

- VII. Patients unable to follow the protocol.
- VIII. Patients with interstitial cystitis.

## Experimental Design

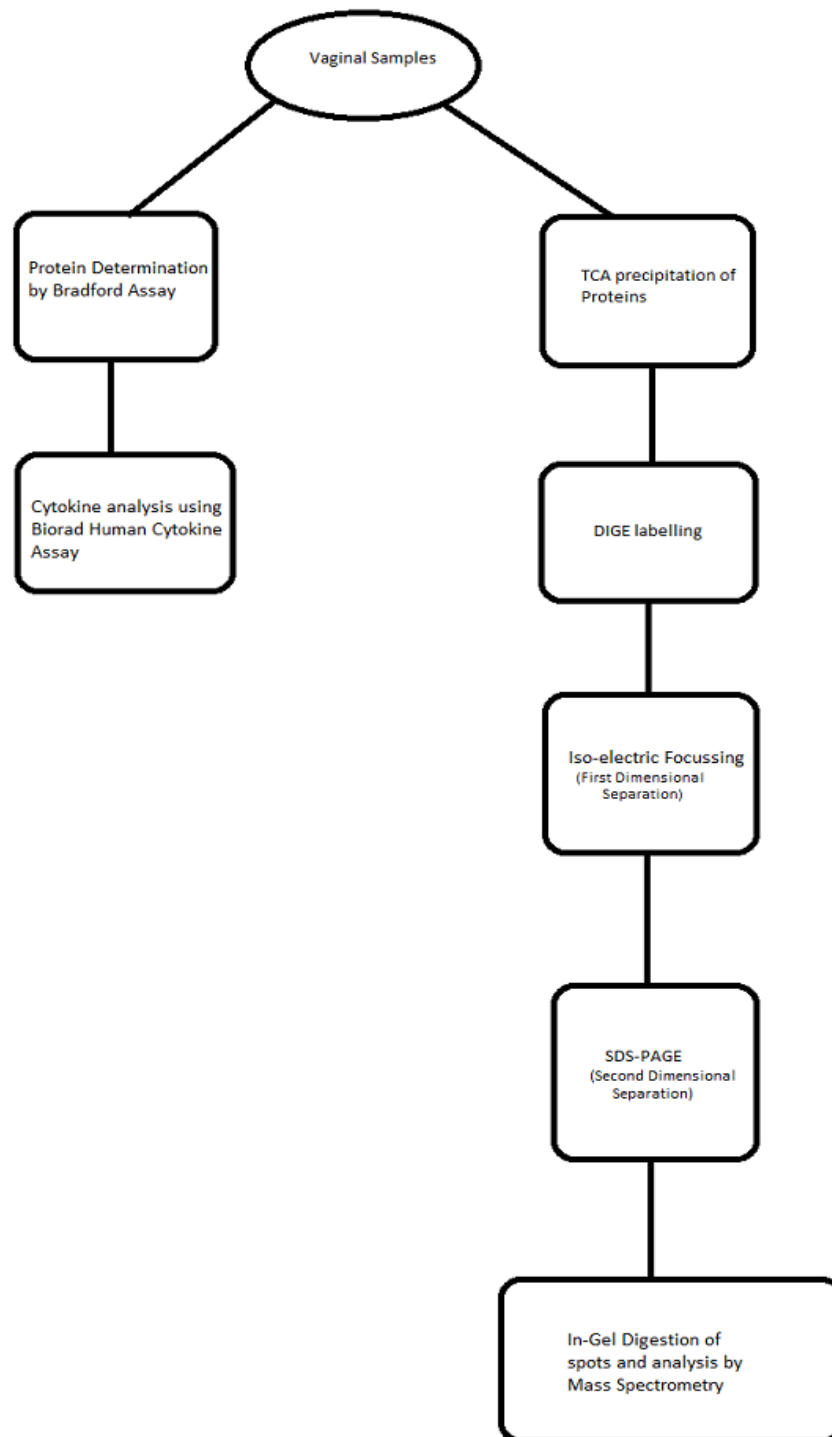


Figure 4: Experimental Design

## **Sample Collection**

Samples were collected in the form of Vaginal Swabs by our collaborating Obstetrics and Gynecological Physicians who have experience with diagnosis and treatment of Vulvodynia. Patients visiting their clinic for the treatment and regular check-ups were used for sample collection under an approved IRB. Vaginal swabs from 15 patients showing active symptoms of vulvodynia were collected and 5 vaginal swabs from patients showing no symptoms of vulvodynia were used as control for the study. Samples were received and stored at -80°C.

## **Cytokine Analysis**

Bio-plex Pro cytokine assay are essentially multiplex immunoassays which make use of magnetic beads to couple the biomarker of interest. This technique is a slight modification of the sandwich ELISA. The antibodies are covalently linked to the beads and bind to the biomarker of interest when treated with the sample. These beads are then washed repeatedly to remove excess protein and a detection antibody is added that binds to the primary antibody, thus completing the sandwich complex. Streptavidin-phycoerythrin conjugate is then added to serve as a fluorescent indicator.

The 96-well plate from the Human Cytokine Kit is first wetted with Assay buffer, Beads solution is then added to the respective wells and washed twice with wash buffer. Samples and controls (50 µl) are loaded into the wells and analyzed in duplicate. The plate is kept for some time for incubation and is washed three times with wash buffer. Detection antibody is added to the wells and incubated for 30 minutes at room temperature. Streptavidin-phycoerythrin conjugate is then added to the wells and

subjected to three washes with wash buffer. The plate is scanned on the Bioplex 200 instrument and statistics computed using One way ANOVA.

### **Trichloroacetic acid (TCA) precipitation of proteins**

Five vulvodynia samples were selected from the pool of 15 samples based on the highest amount of protein content for 2D-DIGE analysis. The protein content was determined using Bradford's test. All five controls were used for this analysis. 200 microliters of vaginal specimen samples and controls was taken into a new microcentrifuge tube. 20 microliters of TCA was added to the samples and controls. The tubes were placed on ice for at least one hour to precipitate proteins. The tubes were then centrifuged and the pellet was rinsed with acid acetone (39 parts acetone: 1 part hydrochloric acid). The tubes were centrifuged and the pellet was resuspended in sufficient lysis buffer (8M Urea, 30 mM Tris and 4% CHAPS) to ensure complete solubilization of the pellet. The pH of this solution was adjusted between 8.5- 9.0 with dilute sodium hydroxide. The pH was checked using a pH paper by spotting 2 microliters of the sample. An internal standard was created using 100 µl of vulvodynia sample and 100 µl of control samples that were to be analyzed on the same 2D gel and were treated in a similar manner.

### **DIGE Labelling of Samples**

Vulvodynia samples (20 µl) were labelled with 1µl of Cy3 dye. 20 µl of each control samples was labelled with 1 µl of Cy5 dye and 20 µl of the pooled samples was labelled with 1ul of Cy2 dye. The tubes were centrifuged for a brief period and kept on ice for 30 minutes in the dark following which 1µl of 10mM lysine was added to stop the labelling reaction. The tubes were kept on ice for 10 minutes. To each tube, 20 µl of 2X

sample buffer was added (Urea 8M, CHAPS 2%, DTT 2%) and kept on ice for 10 minutes. The three tubes were then pooled into a single microcentrifuge tube.

Rehydration buffer (74µl of 8M Urea, 2% CHAPS, 50mM dithiothreitol, 0.2% w/v Bio-lyte® 3/10 ampholytes and trace amounts of bromophenol blue) was added to the pooled tube.

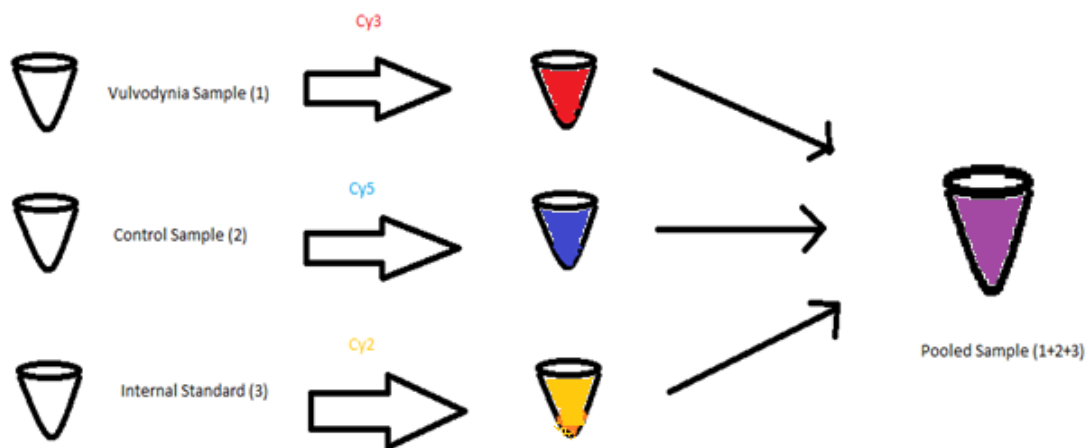


Figure 5: DIGE labeling of samples

### Rehydration of Immobilized pH Gradient (IPG) Strips

Biorad ReadyStrip™ IPG strips 11 centimeters, pH 3-10 was rehydrated using active reabsorption technique. The channel of the focusing tray was loaded with 200 µl of the labelled pooled sample and the strip was faced gel side down to facilitate absorption of the sample into the gel. After one hour, 2 mls of mineral oil was added to the channel to prevent evaporation of the sample and the focusing tray was placed on the PROTEAN IEF Cell. Rehydration was done under active settings at 50V overnight (11-16 hours).

## **Isoelectric Focusing and SDS-PAGE**

Wicks were wetted with 6 µl of nanopure water. The ends of the gel were lifted to place the wetted wick so that it covered the electrode. Wicks are important in absorbing salts and other contaminants that may interfere with first dimensional separation.

Isoelectric focusing was performed under recommended settings in the manual of the Biorad ReadyStrip™ IPG strips. After the run, the strips were held vertically to drain the mineral oil and placed on rehydration trays gel side up. 4 µl of Equilibration buffer 1 (6M Urea, 2%SDS, 0.375 TRIS-HCl pH 8.8, 20% glycerol and 2% w/v DTT was added to the channel in which the strips were placed and the tray was shaken in the orbital shaker for 45 minutes. This buffer was then poured out and 4 mls of Equilibration buffer 2 (6M Urea, 2% SDS, 0.375 M Tris-HCl pH 8.8, 0.5g Iodoacetamide and 20% glycerol) was added and similarly shaken for 30 minutes. The strips were then rinsed with 1X running buffer and sealed with overlay agarose (0.5% agarose, 25mM Tris, 192mM glycine, 0.1% SDS and trace of bromophenol blue) in the well of BIORAD CRITERION GEL.

BIORAD Precision Plus Protein™ Dual Extra Standards Was used as ladder to mark the progress of the gel run.

## **Imaging**

The gels were scanned using 488nm wavelength for Cy2, 532 nm for Cy3 and 635 nm for Cy5. To facilitate visualization of spots the brightness was adjusted accordingly.

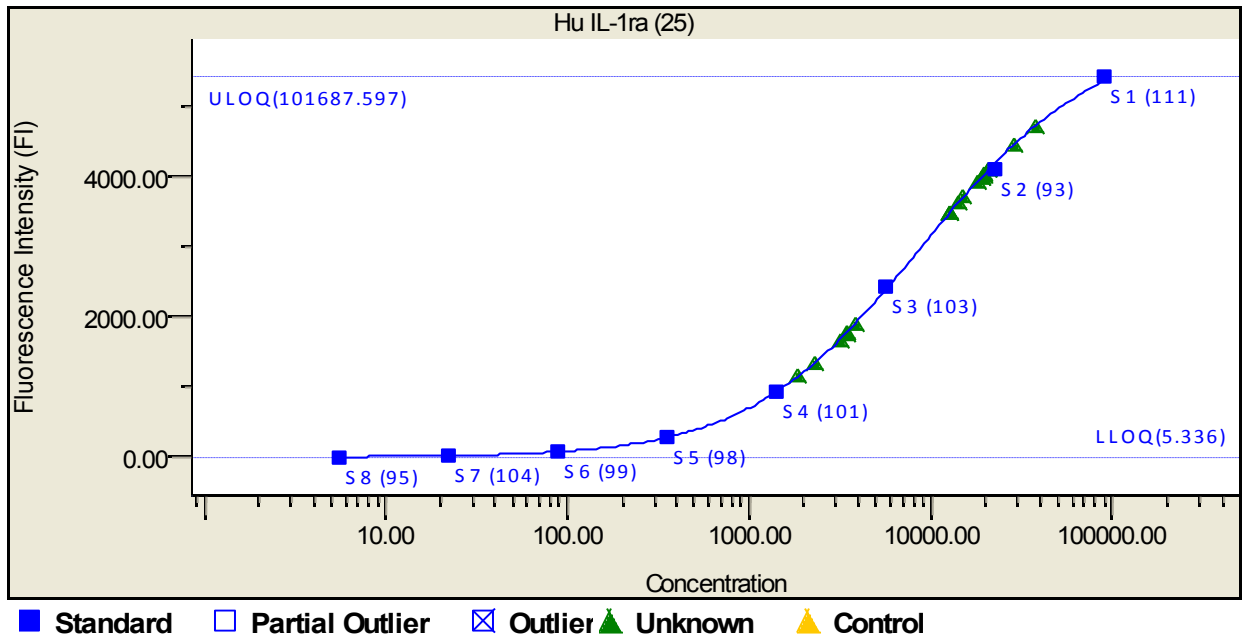
## **Spot picking**

Selected spots were obtained using a gel cutter and placed in a microcentrifuge tube and stored at -80° C.

## RESULTS

### Cytokine analysis of vulvodynia secretions

Vaginal secretions from 15 vulvodynia patients and 5 control patients were subjected to cytokine analysis using the Bio-Rad Bio-Plex Human Cytokine Assay. Overall, 27 cytokines were assayed and the standard curves for each of them were generated during the same run.



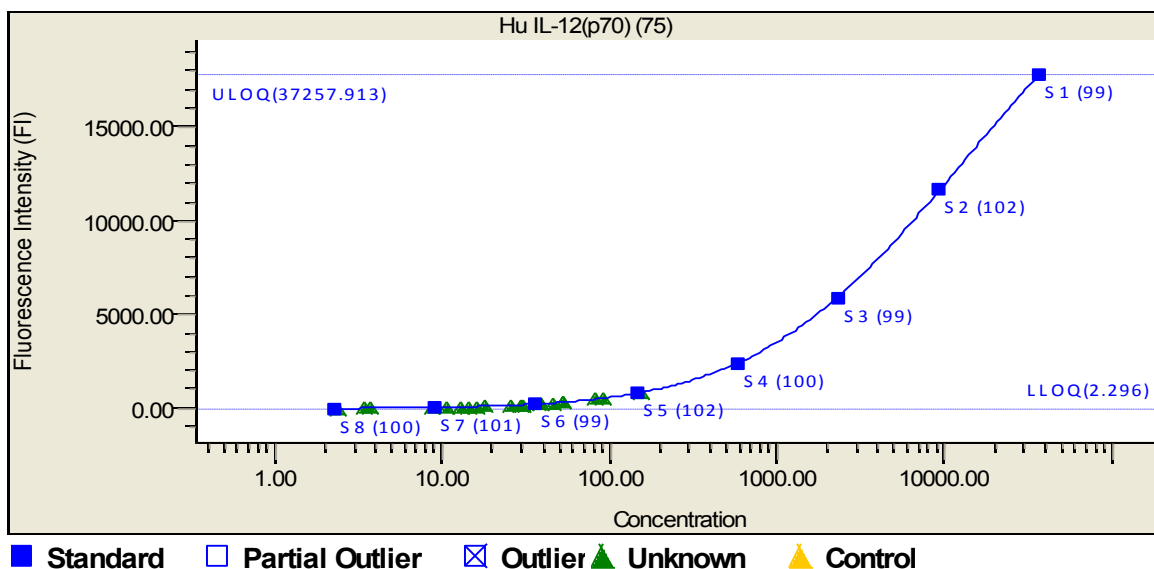
Regression Type: Logistic - 5PL

Std. Curve:  $FI = 3.39674 + (6082.26 - 3.39674) / ((1 + (Conc / 7548.64)^{-0.86524}))^{1.13603}$

FitProb. = 0.8813, ResVar. = 0.2219

Figure 6: Standard Curve of Interleukin-1ra



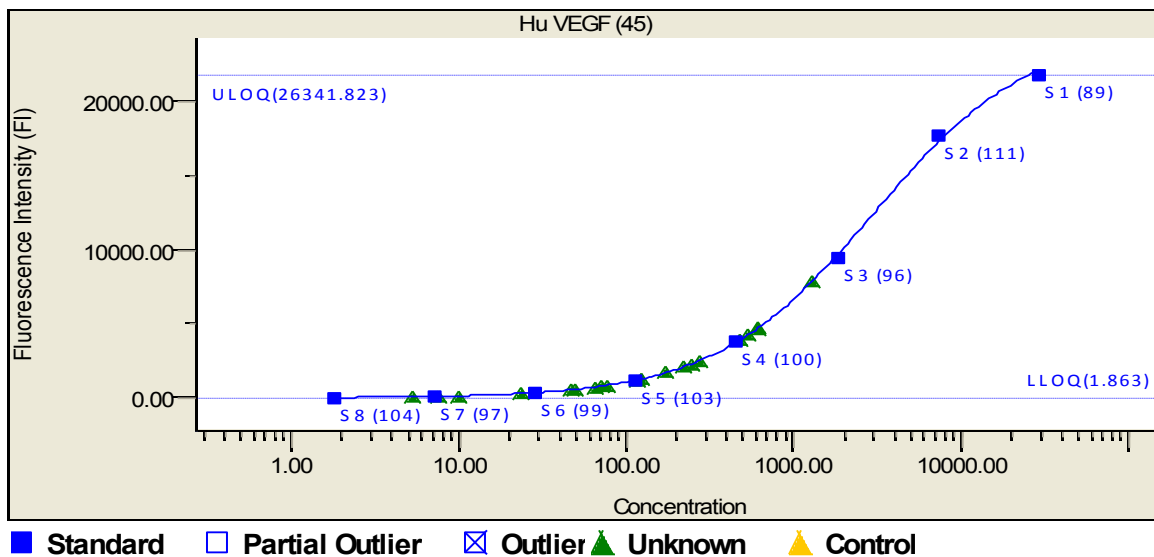


Regression Type: Logistic - 5PL

Std. Curve:  $FI = -0.634235 + (29413.4 + 0.634235) / ((1 + (\text{Conc} / 5075.5)^{-0.546405}))^{1.71983}$

FitProb. = 0.9831, ResVar. = 0.0549

Figure 7: Standard Curve of Interleukin-12 levels



Regression Type: Logistic - 5PL

Std. Curve:  $FI = 9.53232 + (24759 - 9.53232) / ((1 + (\text{Conc} / 3251.83)^{-0.944422}))^{0.943433}$

FitProb. = 0.7774, ResVar. = 0.3662

Figure 8: Standard Curve of VEGF levels

| Cytokine    | Control<br>Mean $\pm$ SEM<br>(pg/ml) | Vulvodynia<br>Mean $\pm$ SEM<br>(pg/ml) | P- Value | Change   |
|-------------|--------------------------------------|---|----------|----------|
| IL-1b       | 83.12 $\pm$ 72.65                    | 103.78 $\pm$ 41.94                      | 0.808    | Increase |
| IL-1ra      | 9557.49 $\pm$ 30292.92               | 96845.95 $\pm$ 17489.63                 | 0.023    | Increase |
| IL-2        | 5.37 $\pm$ 6.05                      | 13.85 $\pm$ 3.50                        | 0.241    | Increase |
| IL-4        | 0.79 $\pm$ 0.56                      | 1.57 $\pm$ 0.32                         | 0.242    | Increase |
| IL-5        | 1.15 $\pm$ 1.86                      | 3.64 $\pm$ 1.07                         | 0.263    | Increase |
| IL-6        | 1.70 $\pm$ 1.56                      | 5.28 $\pm$ 0.90                         | 0.063    | Increase |
| IL-7        | 3.31 $\pm$ 5.85                      | 13.98 $\pm$ 3.38                        | 0.132    | Increase |
| IL-8        | 985.72 $\pm$ 512.48                  | 862.93 $\pm$ 295.88                     | 0.838    | Decrease |
| IL-9        | 6.93 $\pm$ 4.82                      | 15.64 $\pm$ 2.78                        | 0.136    | Increase |
| IL-10       | 28.16 $\pm$ 30.46                    | 96.94 $\pm$ 17.59                       | 0.066    | Increase |
| IL-12       | 31.13 $\pm$ 74.07                    | 228.28 $\pm$ 42.76                      | 0.033    | Increase |
| IL-13       | 5.34 $\pm$ 12.62                     | 34.07 $\pm$ 7.29                        | 0.064    | Increase |
| IL-15       | OR<                                  | 2.37 $\pm$ 1.48                         | -        | Increase |
| IL-17       | 98.22 $\pm$ 21.16                    | 45.61 $\pm$ 12.22                       | 0.045    | Decrease |
| Eotaxin     | 9.32 $\pm$ 8.25                      | 22.90 $\pm$ 4.76                        | 0.171    | Increase |
| FGF basic   | 116.59 $\pm$ 54.27                   | 158.35 $\pm$ 31.33                      | 0.514    | Increase |
| G-CSF       | 40.10 $\pm$ 64.44                    | 147.71 $\pm$ 37.20                      | 0.165    | Increase |
| GM-CSF      | 334.43 $\pm$ 221.05                  | 615.05 $\pm$ 127.62                     | 0.286    | Increase |
| IFN-g       | 15.21 $\pm$ 19.03                    | 45.42 $\pm$ 10.98                       | 0.186    | Increase |
| IP-10       | 190.45 $\pm$ 304.18                  | 391.70 $\pm$ 175.61                     | 0.574    | Increase |
| MCP-1(MCAF) | 33.83 $\pm$ 41.55                    | 99.24 $\pm$ 23.99                       | 0.190    | Increase |
| MIP-1a      | 4.21 $\pm$ 1.28                      | 3.20 $\pm$ 0.74                         | 0.502    | Decrease |
| PDGF-bb     | 17.60 $\pm$ 16.72                    | 62.29 $\pm$ 9.65                        | 0.032    | Increase |
| MIP-1b      | 49.23 $\pm$ 25.10                    | 54.87 $\pm$ 14.49                       | 0.848    | Increase |
| RANTES      | 103.72 $\pm$ 33.20                   | 52.39 $\pm$ 19.16                       | 0.197    | Decrease |
| TNF-a       | 13.33 $\pm$ 10.22                    | 20.33 $\pm$ 5.90                        | 0.560    | Increase |
| VEGF        | 133.38 $\pm$ 345.94                  | 1244.69 $\pm$ 199.73                    | 0.012    | Increase |

Significant differences in the levels of IL-1ra, IL-12, IL-17, PDGF-bb and VEGF was seen between patients with vulvodynia and controls. IL-1ra showed more than 10-fold increase in vulvodynia samples compared to control samples ( $p < 0.03$ ). At the same time, vulvodynia patients demonstrated a 7-fold increase in IL-12 ( $p < 0.04$ ), a 3-fold increase in PDGF-bb ( $p < 0.04$ ) and a 9-fold increase in VEGF ( $p < 0.02$ ) compared to control patients. Also a 2-fold decrease in the levels of IL-17 ( $p < 0.05$ ) was observed in

vulvodynia patients compared to control patients. IL-15 which was not detected in control patients was observed to be up-regulated in vulvodynia patients.

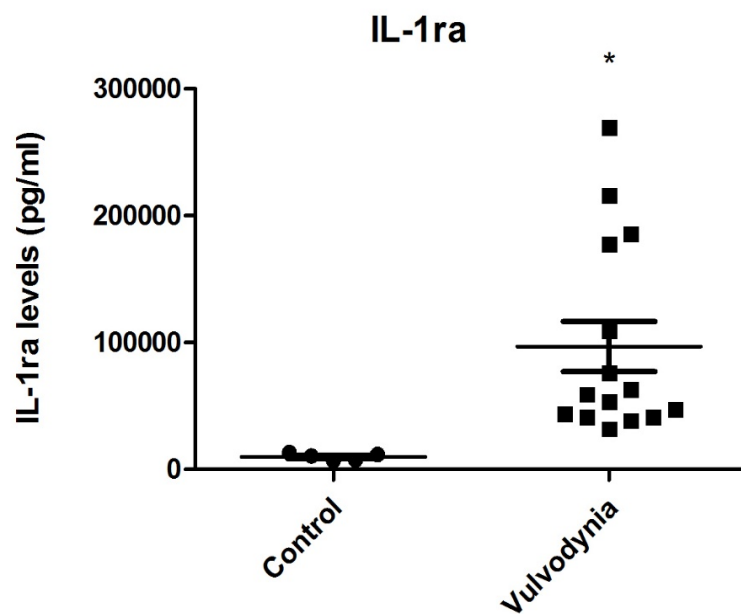


Figure 9: IL-1ra levels in vulvodynia and control patients. The bars represent mean  $\pm$  SEM of IL-1ra levels.

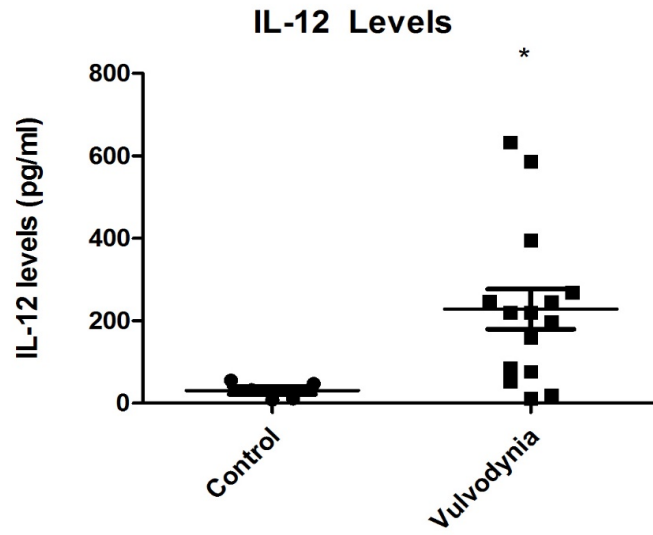


Figure 10: IL-12 levels in vulvodynia and control patients. The bars represent mean  $\pm$  SEM of IL-12 levels.

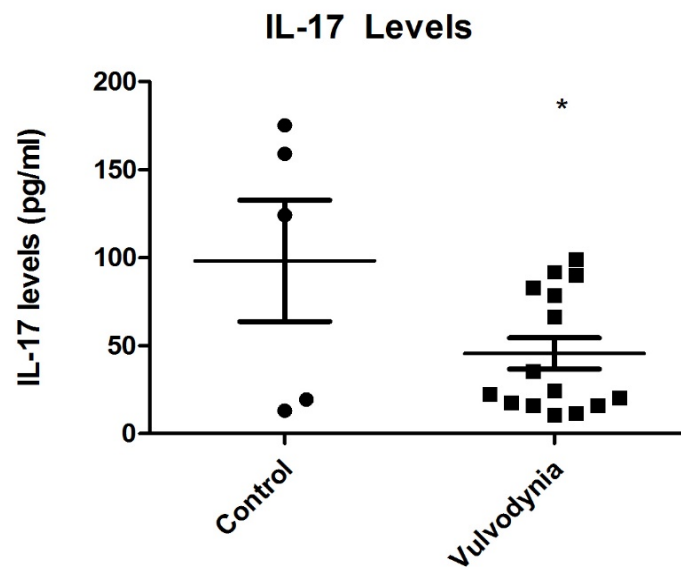


Figure 11: IL-17 levels in vulvodynia and control patients. The bars represent mean  $\pm$  SEM of IL-17 levels.

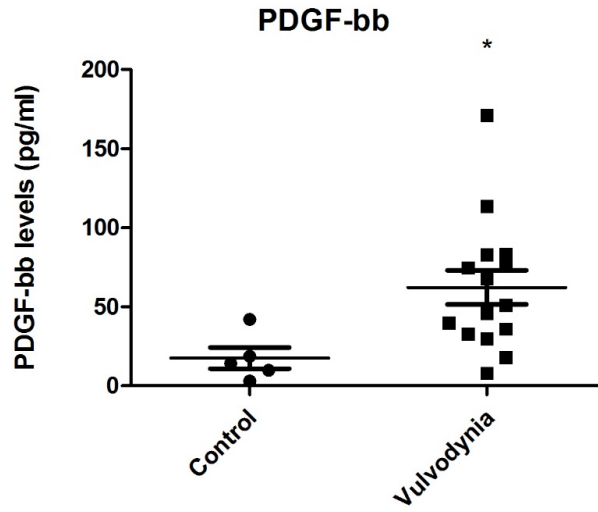


Figure 12: PDGF-bb levels in vulvodynia and control patients. The bars represent mean  $\pm$  SEM of PDGF-bb levels.

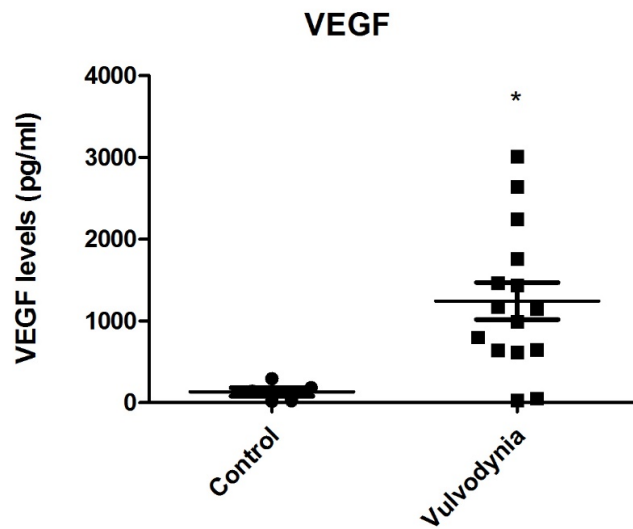


Figure 13: VEGF levels in vulvodynia and control patients. The bars represent mean  $\pm$  SEM of VEGF levels. .

## **2D-DIGE Results**

Cytokine analysis provided an expanded view of specific inflammatory proteins in vulvodynia. However, it is limited to the known quantity and type analyzed by the BioPlex kit. To delve deeper into protein changes in vulvodynia, we chose to use 2D-DIGE analysis of the proteins in the samples. In this method, proteins from disease and treated samples can be differentially labeled with a fluorescent dye prior to running on 2D-Gels. Due to the costs involved and limitations in the control patient samples, we chose to run 5 vulvodynia patient samples against 5 control patient samples by 2D-DIGE. The gels were examined by fluorescence imaging at wavelengths to excite CY2, CY3, and Cy5 dyes attached to the proteins. The samples were precipitated with TCA to concentrate them further. We found that there was a unique pattern associated with the samples from each group (Figure 4, 5, 6). A number of proteins were found to be different between the groups, with some overlapping. A map of each unique protein position was generated, printed and the unique spots pulled from each gel. These spots were then digested with trypsin, cleaned up on C18 ZipTips. We are currently awaiting sequencing by LC/MS.

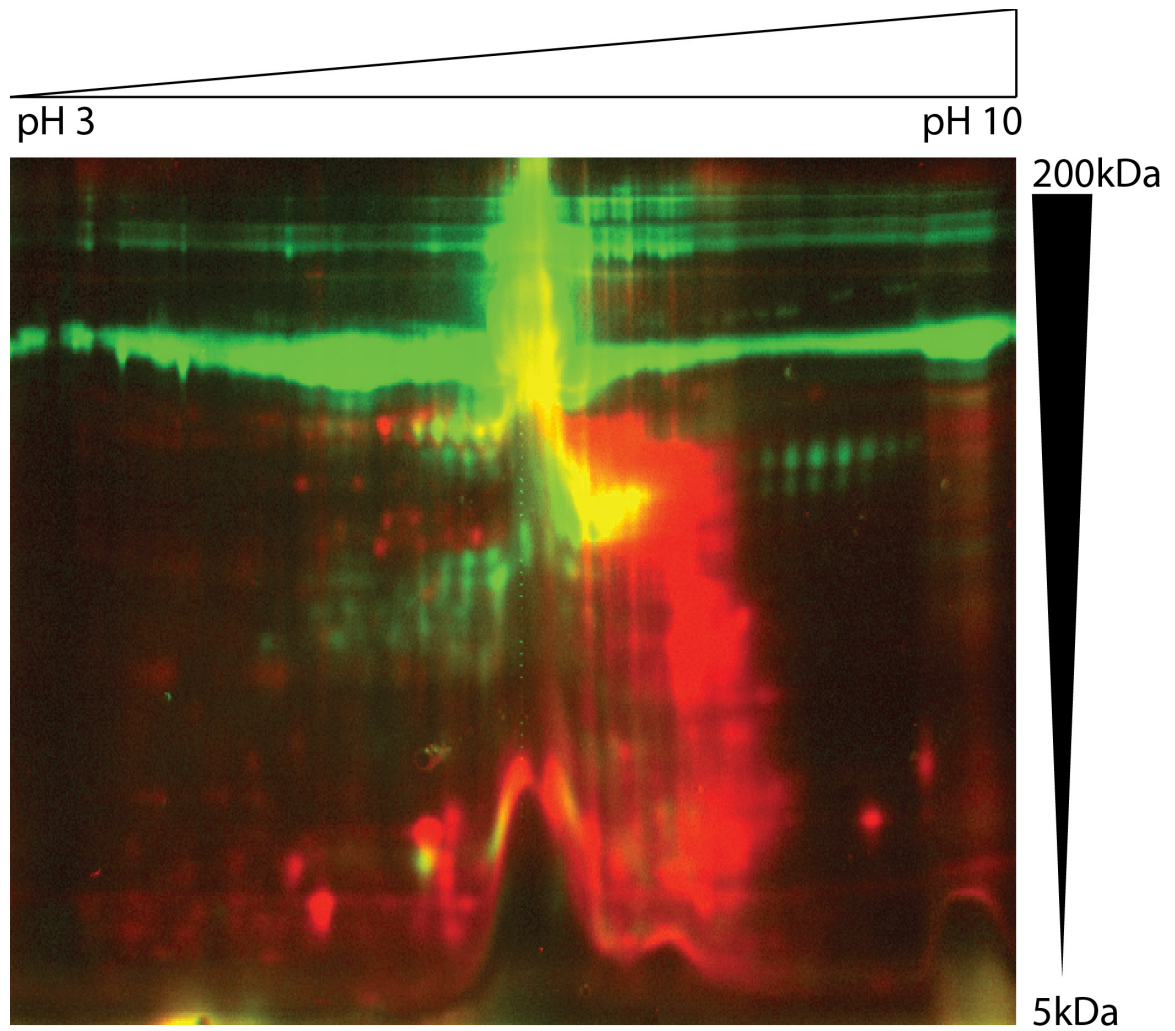


Figure 14: 2D-DIGE labeled vulvodynia and control samples. Control (Green) and vulvodynia samples (Red) were labeled, mixed, and separated by isoelectric point (pH 3-10) in the first dimension and size in the second dimension.

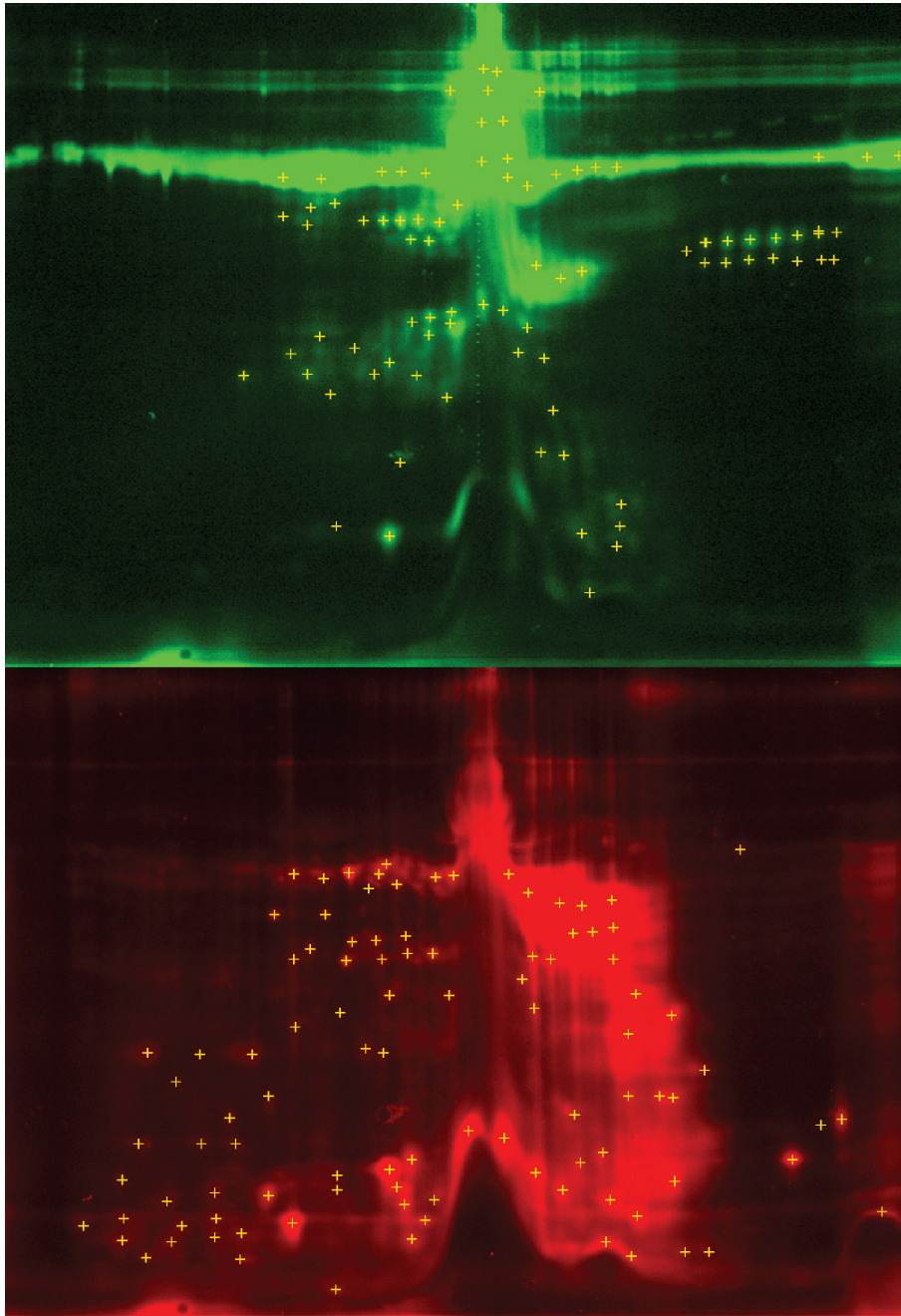


Figure 15: 2D-DIGE Individual Cy3 and Cy5 labelled samples. Unique spots were marked using PDQuest software (Bio-Rad).



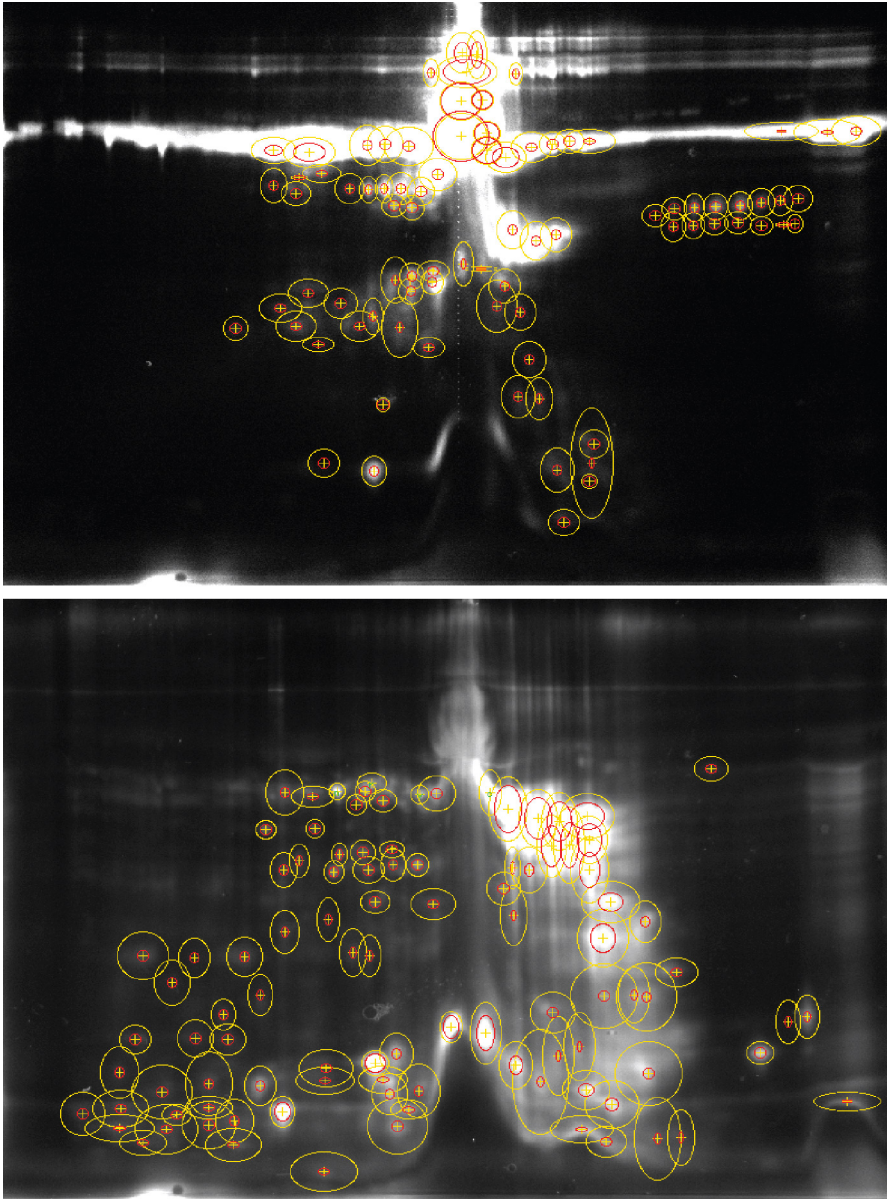


Figure 16: 2D-DIGE Spot Identification. Unique spots were marked using PDQuest software (Bio-Rad) and a protein map generated that was used to direct spot picking. More than 130 gel spots were cut from the gels in this manner.

## **DISCUSSION**

Vulvodynia is a major health problem in the US; as many as 13 million women may suffer from this condition that can lead to morbidity and a reduced quality of life for many women. Vulvodynia is a clinical condition with various poorly defined antecedents. Patients frequently experience: dyspareunia, chronic vulvar irritation, burning, and pain. Some women identify the pain and increased sensitivity as generalized or localized that can be provoked or occur spontaneously (two common diagnostic vulvodynia subtypes: vulvar vestibulitis and vestibulodynia). The experience of chronic pain can consist of a complex interaction of biological, behavioral, sociocultural, and environmental factors. The basic biological process involved in the complexity of this condition is unclear and investigation of such processes involved is vital for constructing a substantive knowledge base. Recent research activities have created the foundation and enhanced our knowledge base, our understanding of the etiology and pathology of this condition is largely incomplete.

What we have learned from published literature that we can certainly exclude in the etiology of vulvodynia is that it is a purely psychological disorder (Boardman and Stockdale 2009; Bohm-Starke 2010) or a direct link to Human Papilloma Virus (Graziottin and Serafini 2009; Gunter et al. 1998). It has been reported there is an initial inflammatory response (Bohm-Starke 2010) which may result in peripheral and central pain sensitization; mucosal nerve fiber proliferation, hypertrophy, hyperplasia and

enhanced systemic pain perception have been reported (Leclair et al. 2011). One of the biochemical mediators of inflammation is cytokines (Omoigui 2007). In women suffering from vulvodynia, the genetic profile includes polymorphisms in genes coding for cytokines, for IL-1 receptor antagonist, for IL-1 beta, and for mannose-binding lectin (MBL) (Gerber, Witkin, Stucki 2008). It is possible that specific proteins could be involved in the etiology of vulvodynia. Female hormones may be significant based on recent reports of an increased progesterone receptor nuclear immunostaining and estrogen receptor alpha expression (Leclair et al. 2011). It is also possible that hormonal changes in addition to cytokines and specific proteins may serve as the causing agent for altering the vaginal flora in vulvodynia.

The intent of our project was to develop new translational research strategies designed to enhance our understanding of the etiology and underlying pathophysiology of vulvodynia. Our hypothesis was that vulvodynia in part elicits an inflammatory reaction that is represented by elevated levels of cytokines. To prove this, we utilized the Bio-Rad Human Cytokine Group 1 Panel that allows us to quantify the levels of 27-different cytokines. In our analysis, we found significant differences among 5 different cytokines between the vulvodynia specimens and control specimens. These are IL-1ra, IL-12, IL-17, PDGF-bb and VEGF. The most marked difference was seen in IL-1ra which showed more than 10-fold increase, followed by VEGF that showed a 9-fold increase, followed by IL-12 that showed a 7-fold increase and PDGF-bb that showed a 3-fold increase in vulvodynia patients compared to healthy controls. Also a 2-fold decrease in the levels of IL-17 was observed in vulvodynia patients compared to healthy controls. Another interesting observation was seen in the levels of IL-15 which was not detected in control

patients but was observed to be up-regulated in vulvodynia patients. These results suggest the involvement of cytokines as a mediator for inflammation in vulvodynia patients. This mediation involves both pro-inflammatory and anti-inflammatory cytokines. The balance between them is crucial in determining the extent of the inflammatory reaction.

High levels of IL-1 has been shown to be involved in the development of many inflammatory and autoimmune diseases of various organs such as joints, gastrointestinal tract, central nervous system and blood vessels (Arend 2002). It is considered to be a prototypic pro-inflammatory cytokine which upregulates the inflammatory reaction (Merhisoussi et al. 2005). IL-1ra is a natural antagonist of IL-1 receptor inhibiting the activity of IL-1 $\alpha$  and IL-1 $\beta$ . It is well known from published literature that levels of IL-1ra is elevated in patients with various inflammatory, pathogenic and post-surgical conditions (Arend et al. 1998). To functionally inhibit the effects of IL-1, it is recommended for IL-1ra levels to be at least 100-fold greater over IL-1 (Arend et al. 1990). Therefore the balance between these two cytokines is important in determining the extent of the inflammatory reaction. Studies utilizing animal models of diseases such as arthritis, inflammatory bowel disease, kidney disease, infectious diseases and many more have shown that either over-production of IL-1 and/or under-production of IL-1ra predisposes to the development of such diseases (Arend 2002). Clinical trials recommend therapeutic administration of IL-1ra to overcome the damaging actions of IL-1. IL-1ra is approved in the treatment of rheumatoid arthritis and can be helpful in other inflammatory diseases (Gabay 2002; Hoffman and Patel 2004). Our study shows a significant increase in the level of IL-1ra in vulvodynia patients suggesting a strong response to an inflammatory

reaction which may be mediated by IL-1. This increase in IL-1ra in such patients may explain the observed low levels of IL-1 in the vulvodynia patients.

IL-12 has been reported to function both as a pro-inflammatory cytokine as well as a key immunoregulatory molecule. Its pro-inflammatory actions include the induction of interferon gamma by T and natural killer cells and enhancing phagocytic and bacteriocidal activity of phagocytic cells (Trinchieri 1998). It is also reported to be involved in the release of other pro-inflammatory cytokines. Its production during the primary phase of infection and inflammation favors differentiation and function of T helper type 1 (Th1) cells and inhibiting the differentiation of T helper type 2 cells (Th2) thereby ensuing antigen-specific immune response (Trinchieri 1998). In our analysis, an increase in IFN- $\gamma$  was seen in vulvodynia patients although this increase was not significant. This increase can be attributed to the increased levels of IL-12. Significant increase in IL-12 levels in vulvodynia patients clearly suggest the involvement of an inflammatory response to this condition.

IL-17 is a key pro-inflammatory cytokine reported to contribute to several inflammatory and autoimmune diseases (Baeten and Kuchroo 2013). Key products of IL-17 are the T helper 17 cells (Th17) as well as cells from the innate immune system such as mast cells and neutrophils (Moran et al. 2011; Kenna and Brown 2012). It induces the expression of other pro-inflammatory cytokine, chemokines, growth factors and other secondary mediators in target cells (Xu and Cao 2010). The detection of decreased levels of this cytokine in vulvodynia patients suggests that IL-17 may be acted upon by other mediators involved in the defense mechanism.

Vascular endothelial growth factor (VEGF) and Platelet-derived growth factor (PDGF) are proteins that play an important role in cell growth and division. Both are involved in angiogenesis. Overexpression of VEGF is a contributing factor in the development of disease, mostly seen in cancer. The role of VEGF has been identified in a large number of disease processes ranging from cancer to autoimmunity, retinopathy, and many more (Kieran, Kalluri and Cho 2012). The function of these growth factors are to repair the damaged tissue. High levels of these seen in vulvodynia may suggest damage of local tissue during the inflammatory reaction.

Hence our analysis can confirm that there is a presence of a strong inflammatory reaction in vulvodynia patients. This reaction first involves the pro-inflammatory cytokines which promotes systemic inflammation followed by the action of anti-inflammatory cytokines which tries to negate the damaging effects of the former.

We are the first group to utilize the 2D-DIGE technology for proteomic studies in vaginal specimens from vulvodynia patients and control patients. This technology allows us to run the vulvodynia sample and the control sample together in one single run thereby minimizing the gel to gel run variation and also reducing the number of gels required to carry out the analysis. In our study we found different spots in the gel between the vulvodynia patients and control patients which suggest there is a change in the proteomic environment in the vulva of vulvodynia patients compared to healthy patients. Some protein spots were specifically observed in vulvodynia patients which may indicate presence of unique and novel proteins in such patients. These spots were identified and were prepared for sequencing analysis by LC-MS. We are currently awaiting the results of the sequencing analysis to help identify what proteins may be involved in this disease.

Identification of such proteins can be useful in identifying markers for detection and therapy.

Limitations of this study includes the use of a small sample size in our analysis. We acquired 15 vaginal specimens from vulvodynia patients and 5 vaginal specimens from healthy control patients. However, the results obtained from our cytokine analysis shows clear significance in the level of 5 cytokines and rules out the possibility of it being due to chance and the presence of any false-positive results. Another limitation includes that the control vaginal specimens were stored in a nutrient liquid containing albumin, which was absent in vulvodynia specimens. This may mask various proteins in the 2D-DIGE analysis of control patients, which may interfere with the interpretation of our results. The proteomic studies were limited to 5 gels due to the sample size of control patients. These 5 samples were paired with vulvodynia samples containing the highest protein content and subjected to 2D-DIGE analysis. This ignores the proteomic studies of the remaining 10 vulvodynia samples in large part due to the cost of the 2D DIGE and cleanup kits required to work with more samples.

Our future plans include to step up this study in a larger number of patients. As we know vulvodynia has various causes, having a larger sample size will allow us to group patients according to their causes and link them with characteristics such as age, race, smoking status, drinking addiction, drug addiction like cocaine users and many such groups. Also it would be interesting to perform longitudinal studies whereby patients are observed from the time of diagnosis and monitored throughout the remainder of their therapy. Carrying out timely studies of such patients and checking the levels of cytokines can be helpful in determining valid biomarkers for vulvodynia.

## APPENDIX

### Reagents and Materials

| Serial No | Name  | Company                   | Catalogue No. |
|-----------|---|---------------------------|---------------|
| 1         | Amersham™ DIGE Fluor, minimal labelling Kit (2nmol) | GE Healthcare             | 28-9345-30    |
| 2         | ReadyStrip™ IPG Strips                              | BIO-RAD                   | 163-2014      |
| 3         | ReadyPrep™ 2-D Starter Kit                          | BIO-RAD                   | 163-2105      |
| 4         | Criterion™TGX™ Precast Gels                         | BIO-RAD                   | 567-1121      |
| 5         | Mineral Oil   | BIO-RAD                   | 163-2129      |
| 6         | N,N,-Dimethyl Formamaide                            | Fisher Scientific         | D119-4        |
| 7         | Lysine  | SIGMA                     | L-5626        |
| 8         | Trichloroacetic acid                                | Fisher Scientific         | A322-500      |
| 9         | Human Cytokine Assay                                | BIO-RAD                   | M50-0KCAF0Y   |
| 10        | Methanol  | Fisher Scientific         | A412-4        |
| 11        | Acetonitrile  | Fisher Scientific         | A955-4        |
| 12        | Criterion Gel™ Blotting Sandwiches                  | BIO-RAD                   | 162-0239      |
| 13        | Albumin, from bovine serum                          | SIGMA Life Science        | A2153-50G     |
| 14        | Acetone   | Fisher Scientific         | A949-4        |
| 15        | Precision Plus Protein Dual Xtra Standards          | Bio-Rad Laboratories Inc. | 161-0377      |
| 16        | Ammonium Bicarbonate                                | Fisher Scientific         | A643-500      |
| 17        | CHAPS   | PIERCE                    | 28300         |
| 18        | Glycine   | Fisher Scientific         | G46-500       |
| 19        | TRIS  | Fisher Scientific         | BP152-500     |
| 20        | SDS   | BIO-RAD                   | 161-0302      |
| 21        | Trypsin from porcine pancreas                       | SIGMA-ALDRICH             | T6567         |
| 22        | Sodium Hydroxide                                    | Fisher Scientific         | S318-3        |



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