MOLECULAR AND IMMUNOLOGICAL MECHANISMS OF EPITHELIAL DISORDERS OF THE VULVA

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Molecular and immunological mechanisms of epithelial disorders of the vulva
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MOLECULAR AND IMMUNOLOGICAL MECHANISMS OF EPITHELIAL DISORDERS OF THE VULVA

Moleculaire en immunologische mechanismen van epitheliale afwijkingen van de vulva

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Entre nós, entre nós
A saudade de amanhã
O mar é tão salgado
Um mar de saudade

C. Branco

Voor mijn vader

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1 INTRODUCTION

1.1 THE VULVA AND VULVAR CANCER

The vulva is the outer part of the female genital tract, bordered by the symphisis pubis, the labiocrural folds and the anus. It consists of the following structures: the mons pubis, the labia majora and minora, the clitoris, the vestibule of the vagina and the urethral orifice (Figure 1). Embryologically all three germ layers are present at the vulva; the cloacal endoderm, the urogenital ectoderm and the paramesonephric mesoderma.[1-2]

Different epithelia, from keratinized squamous epithelium on the labia majora and minora to squamous mucosa in the vestibule, cover the vulva.[3] Dysplastic changes in the epithelium of the vulva are known as Vulvar Intraepithelial Neoplasia (VIN). In the past, various definitions have been used to describe these dysplastic vulvar lesions: morbus Bowen, Queyrat's erythroplasia, carcinoma simplex, bowenoid papulosis, early vulvar cancer, vulvar atypia, hyperplastic dystrophy, carcinoma in situ and dysplasia (graded into mild dysplasia (VIN1), moderate dysplasia (VIN2) and severe dysplasia (VIN3)).[4-6] To simplify this complexity of terms and to enhance diagnostic reproducibility, the International Society for the Study of Vulvovaginal Diseases (ISSVD) defined a new terminology in 2004.[7] We nowadays classify two types of VIN:

1. Usual type VIN (uVIN), which is caused by a persistent Human Papilloma Virus (HPV) infection.
2. Differentiated type VIN (dVIN), which is not associated with HPV, but with chronic inflammatory and/or autoimmune processes, such as lichen sclerosus and lichen planus, involving vulvar mucosa and skin.

Spontaneous regression of VIN lesions has been described, but malignant transformation into vulvar cancer has also been reported.[8-10] Vulvar cancer is the fourth most common gynecological type of cancer.[11] Worldwide the incidence rate is 1 to 2 per 100.000 women, and around 27.000 women are diagnosed each year.[12] In the Netherlands about 200 new cases are identified each year.[13] The pathogenesis of vulvar cancer is not entirely understood. Results from epidemiologic, clinicopathologic and virologic studies suggest two independent pathways leading to vulvar cancer.[14-19] These pathways are visualized in Figure 2. Each pathway results in its own type of vulvar cancer and has its own precursors. Firstly, nonkeratinizing squamous cell cancer of the vulva, with its precursor uVIN; this type of cancer affects younger women and

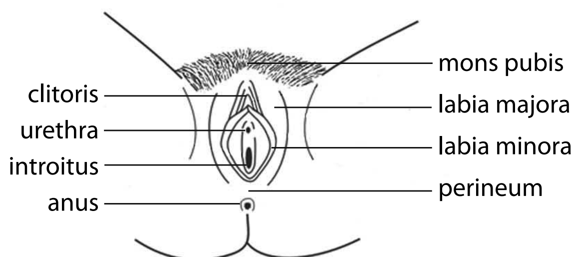

Figure 1. The vulva.
A representation of the vulvar anatomy.

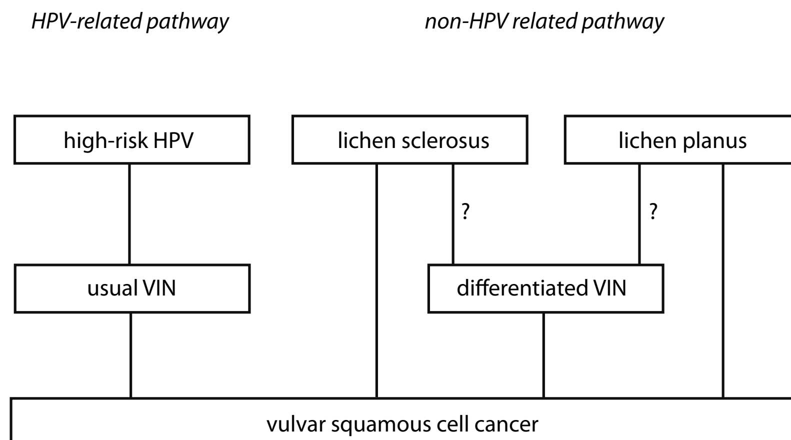

Figure 2. Two pathways leading to vulvar cancer. A representation of the two pathways leading to vulvar cancer, each with their own precursors: usual VIN, lichen sclerosus, lichen planus and differentiated VIN.

is strongly associated with oncogenic HPV types 16 and 18. Nowadays available HPV vaccines will most likely reduce the incidence of this type of vulvar cancer in the future. Secondly, differentiated keratinizing squamous cell cancer of the vulva; this type of cancer is often found in elderly postmenopausal women, with a history of lichen sclerosus, lichen planus or dVIN and has no association with HPV.

1.2 HPV RELATED EPITHELIAL DISORDERS OF THE VULVA

Human papillomaviruses are small double-stranded DNA viruses that infect basal cells of the squamous epithelium. After infection, the virus will replicate in differentiating epithelial cells. For this process different viral proteins are required. Production of these proteins is controlled by the HPV genome, which can be divided into an early and late region. The 'early' region (E) encodes proteins which are expressed early in the viral life cycle. These proteins are involved in control of transcription, replication, and cellular transformation of viral DNA. Furthermore they play a role in uncontrolled cell proliferation. The two most important E proteins in HPV-induced carcinogenesis are the oncoproteins E6 and E7.[20] These oncoproteins bind and inactivate gene products of tumor suppressor genes p53 (mainly by E6) and Rb (by E7).[21-22] In normal cells, p53 and Rb are produced in response to DNA damage, induced by chemicals, radiation or viruses. In reaction to such damage, increased levels of p53 and Rb will regulate the cell cycle. Firstly, high levels of p53 and Rb will induce growth arrest at different checkpoints in the cell cycle to allow DNA repair enzymes to repair damage. Secondly, if DNA damage response fails, they will initiate programmed cell death, also called apoptosis. However, in case of HPV infection, the oncoproteins E6 and E7

can cause inactivation of p53 and Rb, respectively. In this way HPV infected cells are able to escape from protective mechanisms and consequently are more vulnerable to genomic instability caused by DNA damage. This may eventually result in uncontrolled cell growth, one of the hallmarks of cancer.[23]

The 'late'region (L) of the HPV genome contains genes which are expressed late in the viral live cycle and encode the viral capsid proteins (L1 and L2). These proteins are critical to the production of viral capsid and therefore important for the interaction between the virus and the host cell.

Worldwide HPV is the most common sexually transmitted infection, with an 80% life-time risk.[24] Fortunately the majority of these HPV infections (~90%) is cleared by the immune system within one to two years without further adverse consequences for the host.[25] Persistent infections, however, are a well-established risk factor for a large spectrum of epithelial lesions, ranging from benign hyperplasia, caused by low-risk HPV types, to (pre)malignant lesions caused by high-risk HPV types. So far, more than 120 types of HPV are identified. Well-known high-risk HPV types are 16, 18, 31, 33, 35, 45, and 51, and familiar low-risk HPV types are 6,11, 42, 43 and 44.[26]

In chapter of 2 of this thesis we focus on epithelial disorders of the vulva caused by a persistent HPV infection. Here infection with a low-risk HPV type can result in condylomata acuminata, better known as genital warts, whereas infection with a high-risk HPV can cause uVIN. These disorders will be discussed below.

Condylomata acuminata

Low-risk HPV types, like 6 and 11, rarely integrate into the host genome and consequently persistent infection results in benign vulvar disorders like condylomata acuminata.[27] Condylomata are the most common viral sexually transmitted disease in the world. National cumulative prevalence in the United States is estimated to be 6 % of the sexually active population aged 18-59 years.[28-29] Condylomata appear as verrucous or cauliflower-like papules, whereas thick, keratotic warts and small, flat warts may also occur. They are often multifocal and can lead to a variety of symptoms, including anogenital pruritus, burning, vaginal discharge, or bleeding. Histology reveals hyperkeratosis and/or parakeratosis with elongated dermal papillae and koilocytosis (Figure 3A).[30] Most condylomata are self-limiting and regress spontaneously. However, to alleviate symptoms and psychological distress, condylomata are usually treated. Different treatment options are known, including antiproliferative (e.g. podophyllin), immunomodulatory (e.g.imiquimod) and destruction/excision therapies.[30]

Usual type VIN (uVIN)

In contrast to an infection with low-risk HPV, persistent infections with high-risk HPV can cause high grade intraepithelial neoplasia, in the vulva known as usual type VIN (uVIN). The incidence of uVIN is approximately 5 per 100.000 women per year and is increasing.[31-32] This increase is most likely due to the overall rise in HPV infections, but is also influenced by a higher awareness and knowledge of VIN. It is mainly seen in relatively young women, often in their 30s and 40s, and can

lead to a variety of symptoms, like vulvar pruritus, pain, ulceration, dysuria and/or psychosexual dysfunction. Lesions are often multifocal and the appearance can range from hypopigmented to pigmented black, brown, grey or red lesions, which may have a warty, granular or plaque-like appearance.[33-34] Histologically, uVIN is characterized by a thickened epidermis, due to proliferation of atypical squamous cells. These atypical cells have abnormal mitotic figures, and show multinucleation, nuclear hyperchromasia and an increased nuclear-cytoplasmatic ratio (Figure 3B).[33] Standard treatment options for uVIN include surgical techniques (wide local excision, partial vulvectomy or skinning vulvectomy and laser vaporization). More recently, new treatment strategies have been described, like vaccination against the HPV-16 oncoproteins[35] and the use of a topical immune response modifier, imiquimod (Aldara®). [36-38] Imiquimod acts by modifying the immune response by stimulating the production of the pro-inflammatory Th1 cytokines. This response is needed to clear the persistent HPV infection. In contrast to other treatment options, imiquimod is a self-administered treatment. It is generally well tolerated, is less invasive than surgery, relieves itching and pain, and does not influence health-related quality of life, body image, or sexuality.[36] Therefore nowadays, imiquimod is preferred as first-choice treatment in the Netherlands.

If left untreated, uVIN may resolve, persist or progress into vulvar cancer.[8, 39] The percentage of women with untreated uVIN that develops cancer is estimated to be 9%.[40] Risk factors for progression to malignancy are: higher grade of dysplasia at time of diagnosis, age over 40 years, immunocompromised state, tobacco use and a history of lower genital tract neoplasia.[41-42]

1.3 NON-HPV RELATED EPITHELIAL DISORDERS OF THE VULVA

Several cutaneaous conditions have a predilection for the vulva and can cause considerable morbidity. Fortunately, since the introduction of specialized vulvar clinics, where gynaecologists and dermatologists collaborate, more attention is paid to these disorders.[43-47] Two frequently diagnosed disorders at vulvar clinics are lichen sclerosus and lichen planus.[47-49] During follow-up of patients with these disorders, vulvar malignancies are described. Whether or not these malignancies are caused by transformation into dVIN is incompletely understood. In this paragraph lichen sclerosus, lichen planus and dVIN will be further discussed.

Lichen sclerosus

Lichen sclerosus is a chronic inflammatory dermatologic disease predominantly affecting the anogenital region in a relapsing and remitting course. It is much more common in women than in men, and it has a bimodal peak incidence in prepubertal girls and postmenopausal women.[50-52] Although the aetiology is not completely understood, several mechanisms have been suggested, including genetic, autoimmune, hormonal and infectious causes.[53-56] Pruritus and pain are the most frequently reported symptoms,

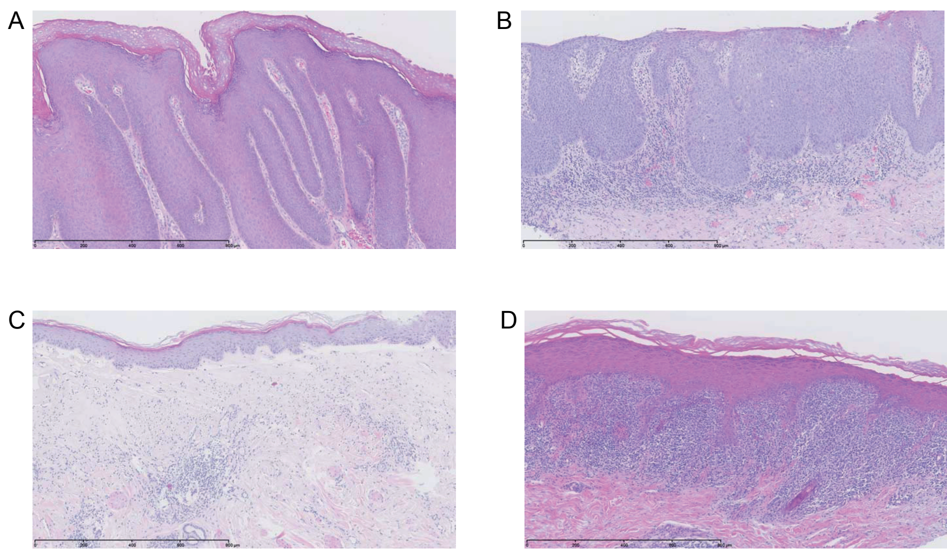

Figure 3. Histopathology of different epithelial disorders of the vulva. Histopathology of **A)** condylomata acuminata, characterized by acanthosis, hyperkeratosis and papillomatosis with elongated dermal papillae and focal koilocytosis, **B)** usual type VIN, showing a thickened epidermis, with atypical squamous cells present through most of the thickness of the epidermis, **C)** lichen sclerosus, characterized by a thinned epidermis with a band of homogenized collagen below the dermo-epidermal junction, deep to which there is a band of inflammatory infiltrate and **D)** lichen planus, characterized by a prominent granular layer, a saw-tooth pattern of the rete ridges and a band-like inflammatory infiltrate in the upper dermis just below the dermo-epidermal junction.

but burning, dyspareunia, dryness, irritation, urinary complaints, constipation and bleeding are also mentioned. Typical lesions may affect the whole vulva including the perianal region, resulting in a figure-of-eight shape, with white, atrophic papules which can be accompanied with hyperkeratotic plaques. In chronic cases, irreversible anatomical vulvar changes can occur.[57] Histologically, the epidermis is thinned with elongated rete pigs. Characteristic is the band-like infiltrate, located under a wide band of homogenized collagen below the dermo-epidermal junction (Figure 3C).[57] To relief symptoms of pain and discomfort and to prevent further anatomical changes, potent topical corticosteroids are the treatment of choice. Unfortunately, however, until now no curative treatments are available for lichen sclerosus. The risk to develop vulvar cancer is estimated to be around 5% and therefore livelong follow-up is advised and all suspicious lesions should be biopsied.[58-59]

Lichen planus

Lichen planus is a chronic inflammatory disease with multiple clinical appearances. In contrast to lichen sclerosus, which only affects the keratinized skin, lichen planus may also involve the mucosa of the vagina and oral cavity. Its cause is unknown,

although current data suggest a T-cell mediated autoimmune mechanism. The disease commonly develops in the sixth decade.[60-62]

The most common clinical variant is erosive lichen planus in which vulvovaginal lesions have an erythematous aspect with white striae known as Wickham's stria.[63] This erosive form results in complaints of itching, burning, pain and dyspareunia.[57, 64-67] Longlasting symptoms can eventually lead to loss of the vulvar and vaginal architecture. The classic histological features of lichen planus are a prominent granular layer, a saw-tooth pattern of the rete ridges and a band-like infiltrate just below the dermo-epidermal junction (Figure 3D).[57] Several treatment options, including both topical and systemic agents are described, but, similar to lichen sclerosus, until now no curative treatments are available for lichen planus.[68] Treatment of first choice is a potent topical corticosteroid, but if not helpful, the use of tacrolimus ointment may be considered.[69-72]

Although the incidence of the development of malignancy from vulvar lichen planus is unknown, cases of vulvar cancer are described.[73-75]

Differentiated type VIN (dVIN)

The clinical and histological diagnosis of dVIN is difficult and it is debatable whether or not it is a separate entity. It is seldom found in an isolated form, but often seen in relation to lichen sclerosus or at the borders of vulvar cancer.[76-78] If it is diagnosed, it is mainly in postmenopausal women with lichen sclerosus. Lesions are often unifocal and can be variable in size and color.[17, 33-34] Due to the fact that most patients are initially diagnosed with lichen sclerosus, the key feature is pruritus.[17] Histologically, dVIN is composed of well-differentiated squamous cells with mild atypia, characterized by nuclear hyperchromasia, mitotic figures and an increased nuclear-cytoplasmatic ratio. In contrast to uVIN, these atypical cells are restricted to the basal and parabasal layers. The superficial layers of the epithelium have normal maturation and do not show koilocytosis.[33] Although the pathogenesis has not yet been clarified, it is believed that dVIN has a higher malignant potential than lichen sclerosus or uVIN.[79-82] This assumption is mainly based on the fact that dVIN is frequently found adjacent to vulvar cancer. To study the malignant transformation from LS and dVIN into vulvar cancer in more detail, van der Avoort et al. studied DNA aneuploidy, a known factor which plays a role in carcinogenesis, in dVIN, lichen sclerosus and vulvar cancer lesions.[83] They observed that DNA aneuploidy was found in 38% of dVIN lesions and in 53% of vulvar cancer lesions, while in contrast no aneuploidy was found in lichen sclerosus. Furthermore, p53 expression was also studied. As discussed before, this is a tumor suppressor gene, which is a key regulator in maintaining genomic integrity. Van der Avoort et al. observed a positive correlation between p53 expression and aneuploidy status, suggesting that dVIN has a higher malignant potential than lichen sclerosus.[83] However, the progression of lichen sclerosus to dVIN and the exact mechanisms of progression into vulvar cancer, remains unclear and further research is needed.

1.4 IMMUNOLOGY

The immune system seems to play an important role in the above described vulvar disorders. Whereas in the HPV related disorders the immune response seems to be suppressed, the immune response appears to be overactive in the lichenoid disorders. To understand the dysfunctional immune response, it is important to understand the working mechanism of a normal immune response. A functional immune response consists of a fast first-line innate immune response, which is followed by a more sustained adaptive immune response.[84] These two responses will be discussed below.

Innate immune response

The innate immune response is the first line of defense in response to a pathogen. This pathogen can be a foreign antigen such as HPV, or an autochthonous. It is a non-specific reaction in which multiple immunocompetent cells, like natural killer cells, mast cells, eosinophils, basophils, macrophages and neutrophils can identify and eliminate different pathogens.[84] Of special importance for the innate immune response are dendritic cells (DCs). DCs are antigen presenting cells situated in tissues at risk for infection, such as skin. There, immature DC's will recognize possible pathogens by pathogen-associated molecular patterns (PAMPs). These PAMPs can bind to toll like receptors (TLRs) present at DCs. TLRs are transmembrane proteins of which so far 13 types have been identified. Each TLR will recognize a specific ligand. For example TLR 7 and 9 recognize PAMPs from viruses, such as HPV.[85] Activation of TLR signaling will result in production of different inflammatory molecules, such as cytokines and type 1 interferons, to induce a functional immune response to attack the pathogen.[86] These processes will induce the transformation of immature DCs into mature DCs. Mature DCs are characterized by the expression of different receptors, like CD80, CD86 and CD40.[87] These receptors are of importance for migration to the lymph node and activation of T cells.[88-89] By presenting the antigen to naïve T cells, DCs are the essential link between the innate and the adaptive immune response.[89-90]

Adaptive immune response

The adaptive immune response, in which T-cells play a central role, regulates the destruction of pathogen infected cells and is of importance for the development of immunological memory. After DCs have presented antigens to naïve T-cells in the draining lymph nodes, these naïve T-cells will differentiate into so called effector cells. There are different types of effector cells: cytotoxic T-cells, T-helper cells and regulatory T-cells (Treg cells).[84]

Cytotoxic T-cells, also called killer T cells, are characterized by the expression of CD8. They recognize virus-infected cells and kill them. T-helper cells express the surface protein CD4 and play an important role in managing the adaptive immune response. Dependent on which response is required to eliminate a pathogen, naïve CD4+ T cells will differentiate in type 1 T-helper cells (Th1), type 2 T-helper cells (Th2), type 17 T-helper cells (Th17) or Treg cells. This differentiation is mainly driven by cytokines produced by DCs in response to pathogens. Generally, a Th1 type response

is more effective against pathogens that replicate intracellularly and a dysregulated Th1 response seems to play a role in autoimmunity. By contrast a Th2 type response is more important in the defense against extracellular pathogens by stimulating B cells to produce antibodies, especially IgE. An overactive Th2 type response is often seen in patients with allergy and asthma. Th17 cells are thought to play a key role in autoimmune disorders, such as multiple sclerosis, psoriasis, rheumatoid arthritis and diabetes.[91] Treg cells play an important role in the regulation of the immune response, mainly by suppressing it. The differentiation and activation of these immune responses are mediated by cytokines.[92] Each Th response has a unique pattern of dominant cytokines, such as IFNγ, IL12 and TNFβ for Th1; IL4 and IL5 for Th2; IL6, TGFβ, IL17A/F for Th17; and TGFβ and IL10 for Treg cells.

In conclusion, locally produced cytokines stimulate DC and T-cell migration, activation and differentiation.[93-94] Therefore, during a successful immune response, the infected squamous epithelium will be invaded by different types of immune cells.

1.5 OUTLINE OF THIS THESIS

The goal of our current investigations is to study the molecular and immunonological mechanisms that play a role in the pathogenesis of the above described different epithelial disorders of the vulva. Therefore, we have addressed several research questions in this thesis.

In **chapter 2** we focus on the mechanisms involved in the etiology of HPV related epithelial disorders of the vulva. In **chapter 2.1** we aimed to investigate to what extent uVIN, caused by a persistent infection with a high-risk HPV type, resembled the characteristics of cancer, namely 1) sustained proliferative signaling, 2) escape from growth suppression, 3) activation of invasion and metastasis, 4) replicative immortality, 5) induction of angiogenesis and 6) resistance to cell death.[23]

The high prevalence of uVIN in immuno-suppressed women, suggests that a good immune response is important for defense against HPV. Therefore, in **chapter 2.2** we studied the immune response in uVIN in depth. The presence of different immune cells and the expression of different cytokines were investigated to clarify the regulation of the innate and the adaptive immune response in uVIN. The aim of **chapter 2.3** was to assess which biological mechanisms were involved in the malignant potential of high-risk HPV infections, in contrast to low-risk HPV infections, resulting in benign disorders.

Chapter 3 focuses on the non-HPV related vulvar epithelial disorders lichen sclerosus and lichen planus. Firstly, clinical characteristics of patients with lichen planus were studied retrospectively, as described in **chapter 3.1**. Because lichen sclerosus and lichen planus are hypothesized to be autoimmune disorders, we focused in **chapter 3.2** on the role of the immune response in both disorders.

The general discussion (**Chapter 4**) summarizes the findings of this thesis and possible future prospects are discussed.

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2 HPV RELATED EPITHELIAL DISORDERS OF THE VULVA

2.1

HPV RELATED VIN: HIGHLY PROLIFERATIVE AND DIMINISHED RESPONSIVENESS TO EXTRACELLULAR SIGNALS

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ABSTRACT

Vulvar Intraepithelial Neoplasia (VIN) is a premalignant disorder caused by human papillomaviruses. Basic knowledge about the molecular pathogenesis of VIN is sparse. Therefore we have analyzed the gene expression profile of 9 VIN samples in comparison to 10 control samples by using genome wide Affymetrix Human U133A plus2 GeneChips. Results were validated by quantitative real-time RT-PCR analysis and immunostaining of a few representative genes (*TACSTD1*, *CCNE2*, *AR* and *ESR1*). Significant Analysis of Microarrays (SAM) showed that 1497 genes were differentially expressed in VIN compared to controls. By analyzing the biological processes affected by the observed differences, we found that VIN appears to be a highly proliferative disease; many cyclins (*CCNA*, *CCNB*, *CCNE*) and almost all prereplication complex proteins are upregulated. Thereby VIN does not seem to depend for its proliferation on paracrine or endocrine signals. Many receptors (for example *ESR1* and *AR*) and ligands are down regulated. Furthermore, although VIN is not an invasive disease, the inhibition of expression of a marked number of cell-cell adhesion molecules seems to indicate development towards invasion. Upon reviewing apoptosis and angiogenesis, it was observed that these processes have not become significantly dysregulated in VIN. In conclusion: although VIN is still a premalignant disease, it already displays several hallmarks of cancer.

INTRODUCTION

Vulvar Intraepithelial Neoplasia (VIN) is a premalignant disorder, characterized by dysplastic changes in the squamous epithelium of the vulva. Recently the International Society for the Study of Vulvovaginal Disease (ISSVD) renewed the classification for squamous VIN, describing two types of VIN: Usual Type VIN (basaloid or warty VIN) and Differentiated Type VIN.[1] Differentiated Type VIN is mostly found in postmenopausal women and is associated with lichen sclerosus. In contrast, Usual Type VIN (undifferentiated) is often diagnosed in premenopausal women and is strongly associated with sexually transmitted Human Papilloma Virus (HPV) infections. In this report, we will focus on HPV-related, Usual Type VIN.

During the last decade VIN has been diagnosed with increasing frequency in relatively young women in western countries. This rise in incidence could be the result of a higher awareness and knowledge of VIN, but is more likely to be due to the overall increase in sexually transmitted diseases, and especially the rise in HPV infections. Around 40% of young, sexually active women are infected with high-risk HPV.[2] Fortunately most women are able to clear the infection, and less than 10% of infected women develop a persistent HPV infection which causes dysplasia of the lower genital tract and might cause VIN.[3] Such a persistent infection with high-risk HPV (mostly HPV16, 18, 31 or 33) may trigger VIN to further develop into invasive vulvar cancer. What percentage of women with VIN eventually develop cancer is difficult to assess, because most patients with VIN will be treated effectively. Van Seters et al. have reviewed data on 3322 published patients with VIN and found 88 untreated patients, of whom eight (9%) progressed to vulvar cancer.[4]

A critical step in the defence against high-risk HPV is a good immune response. Accordingly, being immunocompromised is a risk factor for the development of VIN and subsequent vulvar cancer. For example, VIN is observed more often in women infected with HIV,[5] and in women who are on systemic steroids to treat autoimmune diseases or to prevent rejection after transplantation.[6] Smoking also is a risk factor,[7] because it results in decreased local immune response in the epithelium by reducing the number of intraepithelial Langerhans'cells.[8] For this reason, women with VIN are usually advised to stop smoking.

VIN often presents as multifocal vulvar lesions. The gross appearance as well as the colour of the lesions can vary widely. Most patients are asymptomatic, but when symptomatic the commonest symptoms are pruritus, pain and dysuria.[9] Besides these vulvar lesions, there often also are lesions at cervical, vaginal or perianal sites. Spontaneous regression of VIN lesions has been described, but as previously mentioned, there is no doubt that VIN can progress into vulvar carcinoma. For this reason it is important to treat women with VIN. Standard treatment options for VIN include surgical excision (wide local excision, partial vulvectomy, or skinning vulvectomy) and laser vaporization. Unfortunately recurrences of VIN are common.[10] Extensive surgery, such as vulvectomy, can lead to abnormal anatomy and thereby cause impairment of self-image and sexual function.[11] Recently, in a pilot study, Van

Seters demonstrated the positive effects of treatment with imiquimod, a topical immune response modifier.[12] It was observed that in approximately 30% of cases treatment with imiquimod led to complete remission of the lesions, while another 60% of patients showed a partial remission. Severe vulvar burning after application of ointment was the only main adverse-effect. Van Seters also showed that arousal of HPV16-specific type 1 cellular immunity by induction of local inflammation by imiquimod was involved in regression of Usual Type VIN-lesions.[13] At this moment, the same investigators are conducting investigations into the long-term effects of imiquimod in a randomized clinical trial; results of this study will be published soon (Van Seters et al., submitted).

Treatment options for VIN are few and success rates are less than desired due to the high recurrence rate. In order to be able to develop new treatment modalities for VIN it is important to understand the molecular mechanism behind the disease. Therefore, we have analyzed the gene expression profile of VIN lesions in comparison to matched controls.

MATERIAL AND METHODS

Patient samples

A total of 19 snap frozen vulvar samples (9 high grade (VIN 2 or 3) non-treated VIN biopsies and 10 control biopsies) were studied. All VIN samples were collected previous to any treatment at the start of a randomized clinical trial investigating the effectiveness of 5% imiquimod cream.[14] All samples were reviewed by two pathologists and depending on the the level of cellular disarray they were graded into VIN 1, 2 or 3. The VIN samples were histologically matched to a group of 10 control samples obtained from healthy women visiting a medical center for plastic cosmetic surgery for reduction of the labia minora. From each biopsy 40 cryo-sections of 10 µm were obtained and the first and last section were stained to assess tissue morphology. The remaining sections were used to isolate RNA from. Handling of tissues was done in such a way to minimize variation induced by technical handling.[15] The study design was approved by the Medical Ethical Committee and all women gave written informed consent.

Isolation of RNA and gene expression data analysis

Cryostat sections of vulvar biopsies were homogenized by sonography (Bransom Digital Sonifier, Danbury CT, USA) at 70% of the maximal amplitude, six times 10 seconds at 0°C and RNA was isolated using Trizol (Invitrogen, Life Technologies, Philadelphia, PA). One µg of total RNA was used to prepare antisense biotinylated RNA (www.affymetrix.com). The level and quality of cRNA was measured on the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) and only intact RNA was used. The cRNA was fragmented with the GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA). Hybridization to Affymetrix U133plus2 GeneChips (54,614 probe

sets, representing approximately 47,000 transcripts), staining, washing, and scanning procedures were carried out as described (Affymetrix, Santa Clara, CA). Data were normalized according to the quantile method.[16] After normalization, the intensity values below 30 were set at 30, since our method reliably identifies signals with an average intensity value of 30 or more but does not reliably discriminate values between 0 and 30. Raw and normalized microarray data have been deposited in the GEO repository at NCBI under accession GSE5563.

Data analysis

Cluster analysis: For each probe set, the geometrical mean of the hybridization intensities to that probe set in all samples (both VIN and controls) was calculated. The level of expression of each probe set in every sample was determined relative to this geometrical mean and logarithmically transformed (on a base 2 scale). Genes whose level of expression differed at least 3-fold, in at least one sample, from the geometrical means of all samples (reflecting up- or down-regulation) were selected for the initial cluster analysis using the visualization tool of Omniviz (Omniviz, Maynard, MA) (version 3.8).

SAM analysis: We used Significance Analysis of Microarrays (SAM), implemented in Omniviz to determine genes with significantly differential expression between VIN and control samples. SAM is a statistical method for identifying differentially expressed genes while controlling the overall False Discovery Rate (FDR). FDR is the percentage of genes identified by chance. We have chosen the settings for this analysis in such a way that the total number of falsely identified differential expressed genes was less than one.

Pathway and network analysis: Genes were classified according to biological processes by using Ingenuity Pathway software (Ingenuity® Systems, www.ingenuity.com). Differentially regulated genes between VIN and control were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of the differentially expressed genes were then algorithmically generated based on their connectivity. The final created network is a graphical representation of the molecular relationships between genes and/or gene products. All depicted connections are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. The KEGG PATHWAY database was used to zoom-in at cell cycle genes (www.genome.jp/kegg/pathway.html).

Validation of microarray expression

Part of the validation of micro-array expression data from the vulvar samples was accomplished by RT-qPCR on 2 selected genes: *TACSTD1* and *CCNE2*. RT-qPCR was carried out as previously described.[17] The sequences of primers and probes used in this study are available upon request. Immunohistochemistry for Ki67 (MKI67), AR and ERα (ESR1) was performed on cryosections. The method was essentially similar to that described by Klaassens *et al.*[18]

2.1

Immunohistochemical scoring and statistics

Evaluation of the staining was performed with a computer-assisted imaging system Image J 1.32j (Wayne Rasband, National Institutes of Health, USA). Any nuclear staining regardless of intensity was considered positive. Positive nuclei were counted in one field of vision at x10 magnification. Scoring was defined as the total number of positive nuclear cells per 1 mm^2 of tissue. Statistical analysis was performed using the Student's t-test (2-sided). $p < 0.05$ was considered to be statistically significant. Results are expressed as mean ± SEM. The slides stained with Ki67 antibodies were counterstained with hematoxyline; the slides stained for AR and ER were not counterstained.

HPV detection

Sections from frozen tissue samples were analyzed for the presence of HPV DNA by using a standard $GP5^{+}/6^{+}$ PCR enzyme immunoassay (EIA) followed by reverse line blot analysis, as described previously.[19]

RESULTS

Patient characteristics

The demographic and clinical information about subjects participating in the study is given in Table 1 and supplementary table S1 (http://www.erasmusmc.nl/47393/1584119/1603959/Santegoets). Table 1 shows that the VIN and control groups are comparable with respect to age and tobacco use. As expected, the frequency of cervical pathology (Cervical Intraepithelial Neoplasia (CIN)) was much higher in the VIN group than the control group. Table S1 gives additional clinical data: the control group showed normal histopathology, and VIN was in all cases assessed as Usual Type VIN. HPV type was HPV16 (n = 5), or HPV 33 (n = 3) or 'unknown'(n=1).

Table 1. Patient/control characteristics.

Clinical characteristics	VIN (n=9)	Controls (n=10)
Age (median, range)	39 (33-48)	40 (17-54)
Tobacco use		
Non-smoker	1	2
Ex-smoker	1	4
Light-smoker (1-10 sig/day)	1	0
Moderate smoker (11-20 sig/day)	3	3
Heavy smoker (>20 sig/day)	3	1
History of cervical pathology	7	0
Immunosuppressed	0	0

Cluster analysis

It was established that 2483 probe sets (representing 1736 genes) deviated at least 3-fold, in at least one patient sample, from the geometrical means of all samples (this list of probe sets can be accessed from http://www.erasmusmc.nl/47393/1584119/1603959/Santegoets). This set of genes was used for unsupervised cluster analysis. Performing an unsupervised cluster analysis gives information about how groups are related to one another. As observed in Figure 1A, there are two main clusters, corresponding exactly to the two main experimental groups: to the right the control group and to the left the VIN group.

SAM and pathway analysis

In order to calculate significant differences in gene expression between different experimental groups, so-called SAM analyses were performed. SAM analyses reveal which genes are significantly different between predefined groups. When using SAM to find differentially expressed genes between VIN and control samples, 1497 genes (2049 probesets) were found to be significantly differentially expressed, indicating that there are large differences in gene expression between VIN and control vulvar tissues (this list of genes can be accessed from http://www.erasmusmc.nl/47393/1584119/1603959/Santegoets).

Using quantitative RT-PCR we have confirmed differential expression of 2 selected gene-transcripts (qRT-PCR of *CCNE2* [cyclin E2] and *TACSTD1* [Tumour-Associated Calcium Signal Transducer] (Figure 2).

So far, our analyses showed that there are significant differences between VIN and control tissues. In order to understand the biology behind these differences in gene expression, the genes were categorized according to predefined biological processes, using Ingenuity Pathway software. In Figure 1B we have constructed a combined gene expression network of the seven most significantly regulated processes.

Reviewing this genetic network, a number of fascinating observations were made. The most obvious finding was that a number of important cell cycle genes are highly regulated, which could indicate differential regulation of proliferation between VIN and controls. In order to focus more on regulation of the cell cycle, the results were layered onto the "Cell Cycle Scheme" from the KEGG database (Figure 3). It is clear that many genes involved in regulation of the cell cycle are highly modulated between VIN and controls. Interestingly, not all regulated genes point towards increased proliferation in VIN samples. For example, the high levels of p16 (*CDKN2A*, *p16INK*) inhibit cyclinD expression (*CCND*), which is in contrast to the pronounced upregulation of other cyclins (*CCNA*, *CCNB*, *CCNE*). Therefore, proliferation was also measured using immunostaining of the proliferation marker Ki67 (MKI67). In accordance with our expectation, Ki67 immunostaining was significant higher in VIN as compared to controls (Figure 4*a*).

Another remarkable observation from Figure 3 was that almost all "extracellular space" and "plasma membrane" acting genes were down-regulated in VIN, as compared to controls. This could indicate that paracrine and endocrine regulation of affected dermal cells had become reduced. In support of this hypothesis, it was

A

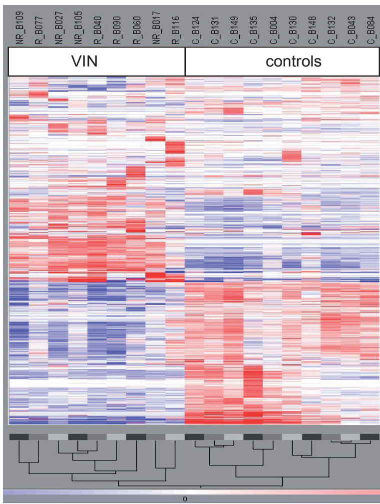
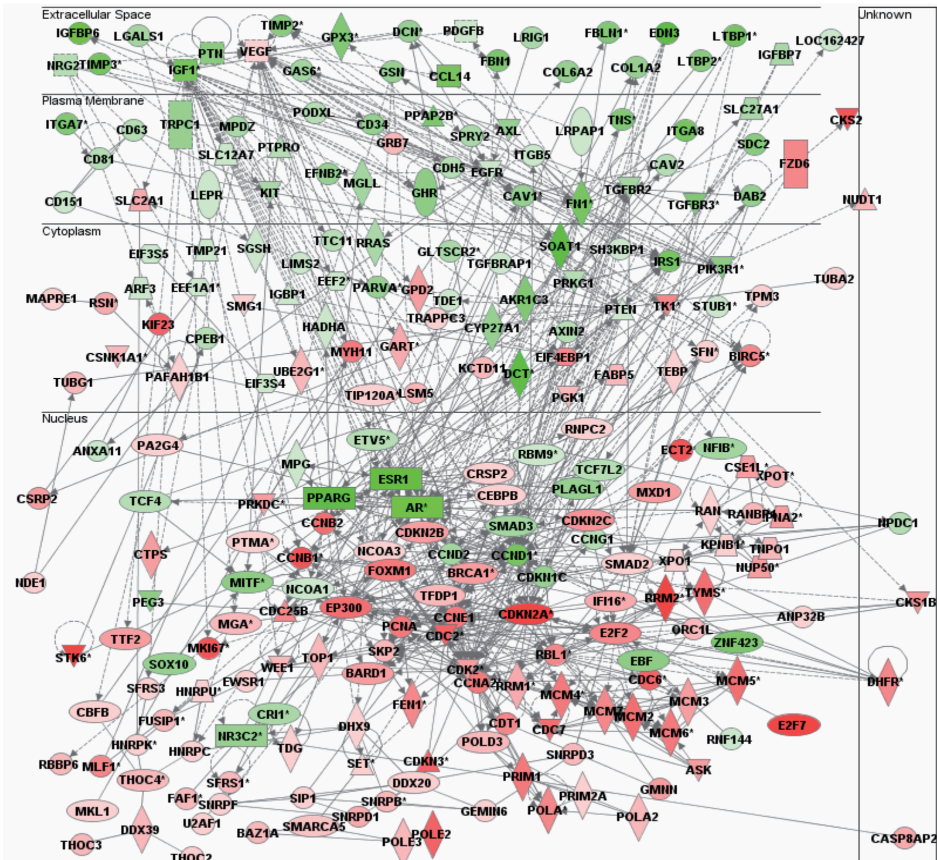


Figure 1. Analysis of gene expression differences between VIN and control. **A**: Cluster analysis of gene expression profiles obtained from VIN (n=9) or matched control tissue (n=10). Each column represents a vulvar sample, coded with a study number (for details about this samples, see the supplementary data on http://www.erasmusmc.nl/47393/1584119/1603959/Santegoets). At the bottom of the figure relatedness is indicated. Red indicates genes with a higher expression relative to the geometrical means, blue indicates genes with a lower expression relative to the geometrical means. **B**: Gene expression network of the seven most significantly regulated biological processes. From top to bottom, genes are acting in extracellular space, plasma membrane, cytoplasm or nucleus. The colors indicate up-regulation (red) or down-regulation (green). The intensity of colors indicates the magnitude of regulation. Relations between genes are indicated with gray lines and functions of genes by the shape of the gene-name boxes. Rectangle: nuclear receptor, square: cytokine, vertical diamond: enzyme, horizontal diamond: peptidase, triangle: kinase, quadrangle: transporter, ellipse: transcription factor, circle: other function.

B



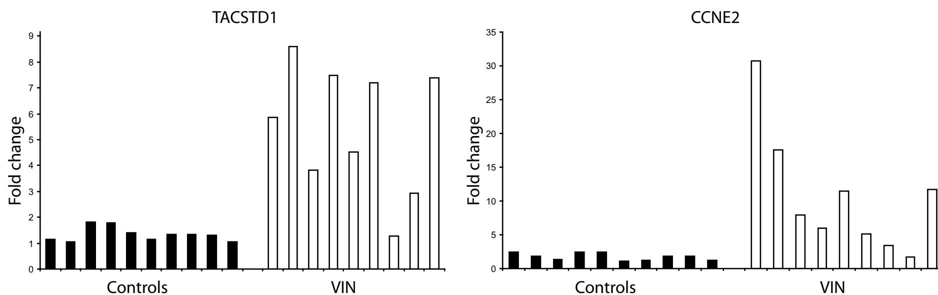


Figure 2. Validation of microarray experiments by quantitative RT-PCR. Validation was accomplished on 2 selected genes TACSTD1 (Tumor associated calcium signal transducer 1) and CCNE2 (Cyclin E2). The Y-axis represents the expression relative to the expression of the household gene β- Actin. The closed bars are values from control patients, the open bars are from VIN patients.

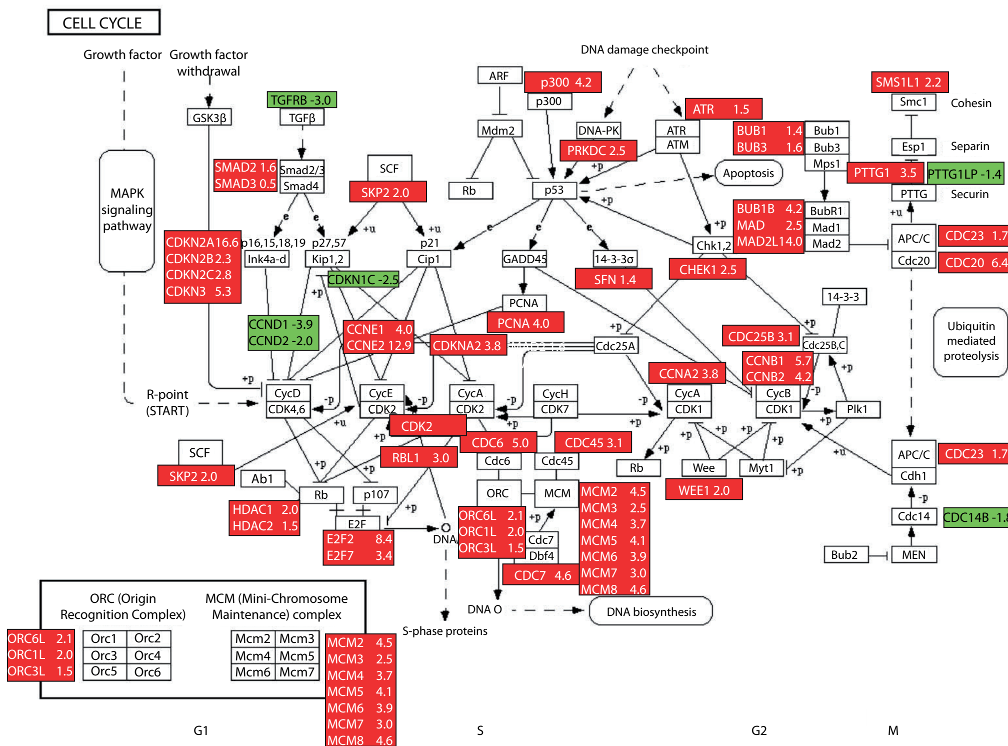


Figure 3. The cell cycle scheme from the KEGG database (www.genome.jp/KEGG/pathway.html) is used to overlay the currently obtained differentially expressed cell cycle genes. Red boxes represent genes with a significantly higher expression (indicated in Fold Change behind the gene name) in VIN compared to controls; green boxes represent genes with a significantly lower expression (indicated in Fold Change behind the gene name) in VIN compared to controls.

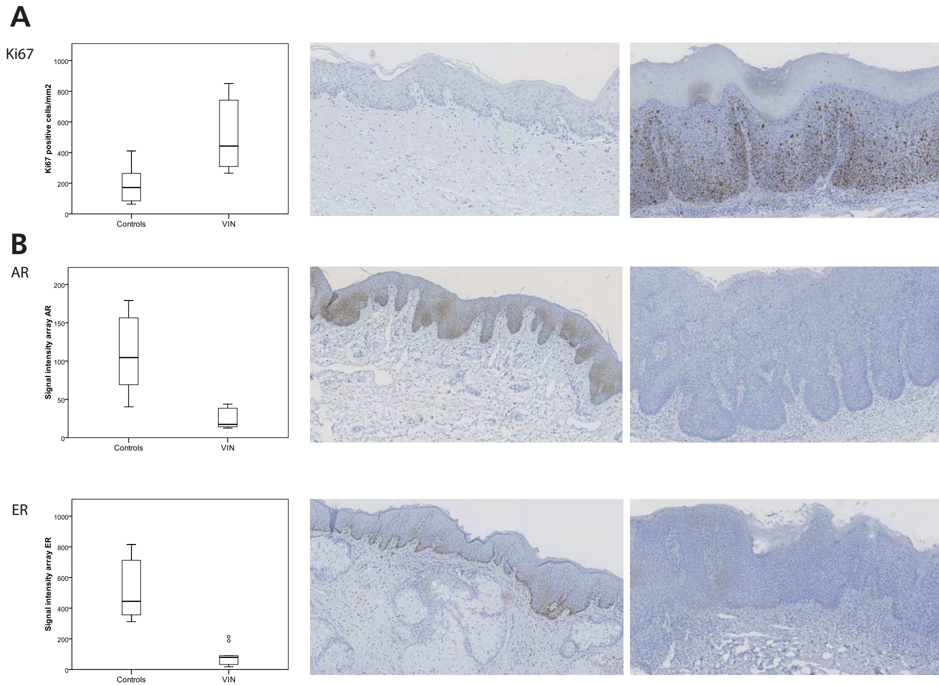


Figure 4. Expression of Ki67, AR and ERα expression in VIN relative to control. Mann-Whitney U- test was performed to calculate significancies. **A:** Ki67 positive cells were counted and represented per mm^2. Median controls: 172, median VIN: 442, p=0.004. **B:** Immunohistochemical staining of AR and ERα was performed and compared to AR and ERα mRNA signal intensities on the array. Median AR controls: 105, median AR VIN: 17, p<0.001.Median ERα controls: 445, median ERα VIN: 79, p<0.001.

observed that two important nuclear steroid hormone receptors (*AR* [androgen receptor] and *ESR1* [estrogen receptor alpha]) were also down-regulated. Because mRNA levels of AR and ER do not always correspond to protein levels, immunostaining for AR and ER was performed. This indeed confirmed pronounced down-regulation of expression at the protein level (Figure 4*b*).

DISCUSSION

Using microarray analysis and subsequent statistical analysis, we identified significant differences in gene expression between high grade VIN and control tissues. Upon analyzing the biological processes affected by the observed gene expression differences, “regulation of the cell cycle” was found to be the most significantly different between VIN and controls. Furthermore, we observed that communication with surrounding cells and tissues seems reduced in VIN.

Increased proliferation and decreased communication are regarded as characteristics of cancer,[20] and, based on the fact that these differences were very pronounced between VIN and controls, we asked ourselves the question to what extent VIN represents cancer. According to Hanahan and Weinberg, cancer is characterized by the following hallmarks: self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, evading apoptosis, tissue invasion and metastasis and sustained angiogenesis. Using our differential gene expression data, we will discuss to what extent VIN has already evolved into vulvar cancer.

Growth signaling: Paracrine and endocrine signaling is important for normal regulation of proliferation. Usually there is a fine balance between signals inducing proliferation and other signals inducing differentiation and consequently inhibiting proliferation. In our VIN tissues the receptors for many hormones are downregulated (Table 2, Receptors). The downregulation of ERα is relevant since estrogens have been described as stimulating antibody responses and to suppressing certain infectious diseases.[21] Our array results show that in women with VIN the expression of ERα in the vulva is very low, so that inflammation may not be suppressed preventing clearance of HPV infection. Thereby, one of the most common complaints among symptomatic women with VIN is itching. It is known that low estrogen levels (or in our case reduced estrogen receptor expression) in postmenopausal women can lead to vulval dryness and itching. Itching can result in a damaged skin, thereby impairing its function of an effective barrier to infection.

Because the ligands for a number of receptors are significantly downregulated (Table 2, Ligands) VIN tissue may be less responsive to endocrine and paracrine factors. Nevertheless, proliferation is increased so at least some proliferative factors must be activated. It is likely that the viral protein E7 has a role to play in induction of proliferation. E7 has been found to associate with *pRb* (retinoblastoma),[22] which causes the release of activated *E2F*, resulting in acceleration of the cell cycle. In VIN this mechanism is further enhanced because of the marked 8.4 fold upregulation of expression of *E2F*. Besides inducing proliferation, the binding of E7 to *pRb* and *p53* also is responsible for the accumulation of genetic mutations. In VIN this is illustrated by the marked upregulation of a great number of genes involved in DNA repair (Table 2, DNA Repair).

Cell cycle regulation: By comparing the expression profiles of normal tissues with those of VIN tissues, a significant difference was observed in the expression levels of many genes that control the cell cycle and proliferation. By performing Ki67 immunostaining we were indeed able to show that proliferation was significantly upregulated in VIN (Figure 4A). It is not surprising that the cell cycle is upregulated in this premalignant disorder, because one of the fundamental steps in cancer pathogenesis is increased proliferation. Reviewing the cell cycle scheme in Figure 4, most of the genes involved in regulating proliferation are upregulated (indicated by the red boxes). However, there is one important exception to this rule: cyclin D. Both cyclin D1 and cyclin D2 are significantly downregulated. This probably results from

2.1

Table 2. Genes involved in different biological processes.

Gene Symbol	Fold change	Gene Title
Receptors		
PPARG	-10,7	PPA receptor, gamma
ESR1	-6,0	estrogen receptor 1
LPHN3	-4,6	Alpha-latrotoxin receptor 3
AR	-4,6	androgen receptor
PDGFRL	-4,4	PDGF receptor-like
EDG2	-3,6	Lysophosphatidic acid G-protein-coupled receptor
IL1R2	-3,4	Interleukin 1 receptor, type II
LOC44068	-3,4	Dopamine D1/D5 receptor
TGFBR3	-3,2	TGF beta receptor III
EDNRA	-3,1	Endothelin receptor type A
MRGPRF	-2,7	MAS-related G protein-coupled receptor, member F
FZD4	-2,7	Frizzled homolog 4
SCARB1	-2,7	Scavenger receptor class B, member 1
NR2F2	-2,7	Nuclear receptor subfamily 2, group F, member 2
GHR	-2,6	growth hormone receptor
FGFR1	-2,6	FGF receptor 1
GPR143	-2,6	G protein-coupled receptor 143
FY	-2,5	Duffy blood group, chemokine receptor
DDR2	-2,5	Discoidin domain receptor family, member 2
FZD7	-2,4	Frizzled homolog 7
GPR124	-2,4	G protein-coupled receptor 124
NR3C2	-2,3	Nuclear receptor subfamily 3, group C, member 2
AXL	-2,3	AXL receptor tyrosine kinase
PTPRM	-2,2	Protein tyrosine phosphatase, receptor type, M
EDNRB	-2,2	Endothelin receptor type B
KIT	-2,0	Steel Factor Receptor
PGRMC2	-2,0	Progesterone receptor membrane component 2
CXADR	2,3	Coxsackie virus and adenovirus receptor
PAQR4	2,5	Progestin and adipoQ receptor family member IV
FZD6	3,0	Frizzled homolog 6
NETO2	4,8	neuropilin (receptor for VEGF165) -like 2
Ligands		
EDN3	-12,1	Endothelin 3
APOE	-8,1	Apolipoprotein E
SLIT2	-4,1	Slit homolog 2 (Drosophila)
IGF1	-4,0	Insulin-like growth factor 1 (somatomedin C)
CCL14/15	-3,8	Chemokine (C-C motif) ligand 14/15
DKK3	-3,2	Dickkopf homolog 3 (Xenopus laevis)
PTN	-2,8	Pleiotrophin (heparin binding growth factor 8)
EFNB2	-2,5	Ephrin-B2
GAS6	-2,1	Growth arrest-specific 6
CORT	3,5	Cortistatin
DNA Repair		
NUDT1	2,0	Nudix-type motif 1
RAD21	2,0	RAD21 homolog
MBD4	2,1	Methyl-CpG binding domain protein 4
KUB3	2,1	Ku70-binding protein 3
BRCA2	2,1	Breast cancer 2, early onset
RAD54B	2,1	RAD54 homolog B
RAD54L	2,2	RAD54-like
DCLRE1B	2,2	DNA cross-link repair 1B
BLM	2,2	Bloom syndrome
EXO1	2,4	Exonuclease 1
PRKDC	2,5	Protein kinase, DNA-activated, catalytic polypeptide
CHEK1	2,5	CHK1 checkpoint homolog
FANCL	2,6	Fanconi anemia, complementation group L
BRCA1	2,6	Breast cancer 1, early onset
MSH6	2,7	MutS homolog 6
MSH2	2,8	MutS homolog 2

Table 2. *Continued*.

Gene Symbol	Fold change	Gene Title
RAD51	2,9	RAD51 homolog
BRIP1	3,8	BRCA1 interacting protein C-terminal helicase 1
RAD51AP1	4,2	RAD51 associated protein 1
Pre-replicative Complex		
ORC1L	2,0	Origin recognition complex, subunit 1
ORC6L	2,1	Origin recognition complex, subunit 6
MCM3	2,5	Minichromosome maintenance deficient 3
CKS1B	2,7	CDC28 protein kinase regulatory subunit 1B
MCM7	3,0	Minichromosome maintenance deficient 7
CDC45L	3,1	Cell division cycle 45
MCM4	3,7	Minichromosome maintenance deficient 4
MCM6	3,9	Minichromosome maintenance deficient 6
MCM5	4,1	Minichromosome maintenance deficient 5
MCM2	4,5	Minichromosome maintenance deficient 2
CDC7	4,6	Cell division cycle 7
MCM8	4,6	Minichromosome maintenance deficient 8
CDC6	5,0	Cell division cycle 6
CKS2	6,1	CDC28 protein kinase regulatory subunit 2
Apoptosis		
BCL2L11	2,2	BCL2-like 11 (apoptosis facilitator)
CASP8AP2	2,2	CASP8 associated protein 2
BCL2L10	4,5	BCL2-like 10 (apoptosis facilitator)
Extracellular Proteases and Protease-Inhibitors		
A2M	-2,0	Alpha-2-macroglobulin
TMPRSS4	4,1	Transmembrane protease, serine 4
Adhesion		
ASAM	-10,0	Adipocyte-specific adhesion molecule
SLIT2	-4,1	Slit homolog 2
ITGA8	-3,8	Integrin, alpha 8
FN1	-3,7	Fibronectin 1
EPDR1	-3,6	Ependymin related protein 1
PGM5	-3,4	Phosphoglucomutase 5
SGCE	-3,3	Sarcoglycan, epsilon
ITGA7	-3,2	Integrin, alpha 7
DCHS1	-3,1	Dachsous 1
TNS	-3,0	Tensin
LAMB1	-2,8	Laminin, beta 1
SSPN	-2,7	Sarcospan
SCARB1	-2,7	Scavenger receptor class B, member 1
DST	-2,7	Dystonin
ITGA9	-2,6	Integrin, alpha 9
CD34	-2,5	CD34 antigen
PCDHGC3	-2,5	Protocadherin gamma subfamily C
COL6A2	-2,5	Collagen, type VI, alpha 2
PARVA	-2,5	Parvin, alpha
CAV1	-2,5	Caveolin 1
DDR2	-2,5	Discoidin domain receptor family, member 2
CAPN3	-2,5	Calpain 3
FEZ1	-2,1	Fasciculation and elongation protein zeta 1
CD99	-2,1	CD99 antigen
GAS6	-2,1	Growth arrest-specific 6
TROAP	2,1	Trophinin associated protein (tastin)
ITGB3BP	2,3	Integrin beta 3 binding protein
SSX2IP	3,3	Synovial sarcoma, X breakpoint 2 interacting protein
Angiogenesis		
TIMP3	-3,7	Tissue inhibitor of metalloproteinase 3
PTN	-2,8	Pleiotrophin (heparin binding growth factor 8)
TIMP2	-2,8	Tissue inhibitor of metalloproteinase 2
CD34	-2,5	Marker for angiogenesis
ANG	-2,3	Angiogenin
FSTL1	-2,1	Follistatin-like 1

a very pronounced (17-fold) upregulation of the cyclin-dependent kinase inhibitor 2A (*CDKN2A*, *$p16^{INK4a}$*). Upregulation of *$p16^{INK4a}$* has been described in VIN[23-25] and is thought to arise from interaction of the viral protein E7 with Rb, thus inhibiting Rb function, which results in upregulation of *$p16^{INK4a}$*. Although p16 upregulation is a very sensitive marker for VIN, it does not result in down-regulation of the cell cycle because downregulation of its downstream effectors cyclinD1 and cyclinD2 is successfully neutralized by upregulation of cyclinE1, cyclinE2, cyclinA2, cyclinB1 and cyclinB2. Interestingly, cyclinD1 is upregulated in many cancers, including vulvar cancer,[26] which could indicate that cyclinD1 can play a role in progression of VIN to an invasive tumor.

Apoptosis: Under normal physiological conditions, whenever proliferation is increased, apoptosis also increases. However, in cancer the situation is different; usually apoptosis is gradually becoming inhibited. Upon verifying whether there is pronounced reduction in apoptosis, a number of apoptosis related genes could be identified on the basis of the fact that they are also involved in regulating proliferation or DNA repair (for example: *histone deacetylase* (2-fold up), *E1A binding protein p300* (4.2-fold up), *BRCA1* (2.6-fold up)). When we concentrate on genes that are core to the apoptotic pathway, pronounced regulation was only observed for a small number of genes (Table 2, Apoptosis). These results indicate that apoptosis may not be significantly inhibited in VIN. In line with these expectations, upon measuring apoptosis using TUNEL, it was shown that apoptosis was increased slightly rather than reduced in VIN (data not shown).

Invasion: The invasive potential of cells is enhanced when extracellular proteases are induced, inhibitors of proteases are inhibited and when the expression of cell-cell adhesion molecules is reduced. Since VIN is not an invasive disease, as established in our samples by pathological revision, changes in the expression of genes involved in extracellular proteolysis or cell-cell adhesion would not be expected. For proteases and protease-inhibitors this is true; only one extracellular protease was upregulated and only one protease-inhibitor down-regulated (Table 2, Extracellular Proteases and Protease-Inhibitors). For the cell-cell adhesion molecules, the situation is different: 24 adhesion molecules were significantly down regulated while only 3 were found to be upregulated. These results seem to indicate that high grade VIN, although not an invasive disease, already displays some characteristics suggesting invasion. It will therefore be of considerable interest to compare our VIN data with data obtained from very early vulvar cancer lesions in order to identify specifically those changes involved in invasion.

Angiogenesis: The development of new blood vessels (angiogenesis) is essential for the development, progression, and metastasis of growing malignant tumors. Vascular endothelial growth factor (*VEGF*) is important in this process. In VIN we have observed a small 1.6 fold upregulation of *VEGF*, suggesting the creation of an environment supplying premalignant lesion with oxygen and nutrients. However, regulation of other genes acting in the angiogenic pathway is not pronounced,

and angiogenin and pleiotrophin, both known to stimulate angiogenesis, are even downregulated (Table 2, Angiogenesis). Upon reviewing angiogenesis in situ, using the CD31 and CD34 markers on tissue sections, we could confirm that angiogenesis was indeed not regulated in VIN as compared to controls (data not shown).

In summary, high grade VIN appears to be a highly proliferative disease, which does not seem to depend on paracrine or endocrine signals for its proliferation. Furthermore, although VIN is not an invasive disease, the inhibition of expression of a marked number of cell-cell adhesion molecules seems to suggest development towards invasion. Upon reviewing apoptosis and angiogenesis, it was observed that these processes are not significantly dysregulated in VIN.

2.1

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2.2

REDUCED LOCAL IMMUNITY IN HPV-RELATED VIN: EXPRESSION OF CHEMOKINES AND INVOLVEMENT OF IMMUNOCOMPETENT CELLS

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ABSTRACT

Usual type VIN is a premalignant disorder caused by persistent HPV infection. High prevalence of VIN in immuno-suppressed women suggests that a good innate and adaptive immune response is important for defense against HPV. Here, we explored expression levels of chemokines and related these to the presence or absence of immuno-competent cells (dendritic and T-cells) in affected (HPV-positive VIN) and non-affected (HPV-negative) vulvar tissues from the same patients.

Combining microarray data with quantitative real-time RT-PCR, it was observed that several important chemokines were differentially expressed between VIN and control samples (up-regulation of *IL8*, *CXCL10*, *CCL20* and *CCL22* and down-regulation of *CXCL12*, *CCL21* and *CCL14*). Furthermore, an increased number of mature dendritic cells ($CD208^+$) seemed to be bottled up in the dermis, and although a T-cell response (increased $CD4^+$ and $CD8^+$ cells) was observed in VIN, a much larger response is required to clear the infection.

In summary, it seems that most mature dendritic cells do not receive the proper chemokine signal for migration and will stay in the dermis, not able to present viral antigen to naive T-cells in the lymph node. Consequently the adaptive immune response diminishes, resulting in a persistent HPV infection with increased risk for neoplasia.

INTRODUCTION

During the last decade Vulvar Intraepithelial Neoplasia (VIN), a premalignant disorder, has been diagnosed with increasing frequency in relatively young women in western countries.[1] This rise in incidence is presumably due to the rise in Human Papilloma Virus (HPV) infections. Nowadays, more than 100 types of HPV are identified and the life-time risk for infection with one of these types is around 80%.[2-3] The different types of HPV are subdivided into low-risk (non-oncogenic, e.g. HPV 6 and 11) and high-risk HPV (oncogenic, e.g. HPV 16, 18 and 33). Around 40% of young, sexually active, women are infected with the latter, a high-risk HPV.[4] Fortunately, most women are able to clear this infection, and less than 10% of infected women develop a persistent HPV infection, which is one of the leading causes of preneoplastic and neoplastic lesions in the female genital tract, including VIN.[5]

The host immune response is of critical importance in determining progression or regression of HPV related VIN, since being immuno-compromised is a risk factor. For example, VIN is observed more often in women infected with HIV, and in women who are on systemic steroids to treat autoimmune diseases or to prevent rejection after transplantation.[6-7] Smoking is also a risk factor, because it results in a decreased local immune response in the epithelium by reducing the number of intraepithelial Langerhans cells.[8-9]

After infection, the host develops a virus-specific cell-mediated immune response, which in many instances, will lead to clearance of the virus within one year.[10] This immunological defense system consists of two components, namely a fast first-line innate immune response, which is followed by a more sustained adaptive immune response.

The innate immune response is initiated immediately or within hours after the first exposure to the virus. It is a non-specific reaction in which multiple immunocompetent cells, like natural killer cells (NKs), mast cells, eosinophils, basophils, macrophages and neutrophils are involved. Of special importance for the innate immune response are dendritic cells (DCs). These cells are responsible for the first recognition of viral antigens by toll like receptors (TLRs) at their cell surface. CpG-rich regions in viruses, including HPV, are detected primarily by TLR7 and TLR9.[11]

The adaptive immune response, in which T-cells play a central role, regulates the destruction of infected cells. After DCs have presented viral antigens to naive T-cells in the draining lymph nodes, these naive T-cells will differentiate into so called effector cells. There are three types of effector cells: T-helper cells, cytotoxic T-cells and regulatory T-cells (Treg cells) (respectively identified by the surface markers CD4, CD8 and CD25/HLA-DR). During a successful response to a viral infection, the infected squamous epithelium will be invaded by large infiltrates of T-cells.

For the immunological defense system a specific group of cytokines, namely chemokines (cytokines with chemotactic activities) are important. Locally produced chemokines (such as *CXCL12*, *CCR1*, *CCR2*, *CCR5*, *CCR7*) stimulate DCs and T-cell migration to and from affected tissue. Studies describing this trafficking of

immunocompetent cells in VIN are scarce, but nevertheless it is established that large numbers of T-cells, both T helper-cells (CD4$^+$) and cytotoxic T-cells (CD8$^+$), are present in HPV related VIN.[12-13] Recently, our group investigated the distribution patterns of immunocompetent cells in VIN lesions compared to control tissue. It was observed that DCs and T-cells specifically migrated towards the dermis of a VIN lesion, suggesting that the cellular-immune response upon viral HPV infection occurs mainly in the dermis.[14]

2.2

The concept that the immune response in HPV related VIN seems to be insufficient has lead to new treatment options in which the immunomodulator imiquimod plays a role. Imiquimod binds to TLR7 at the surface of DCs and induces plasma DCs to express and secrete multiple cytokines, such as TNF-α, type-1 IFN and IL-12.[15] Recently, our group performed a randomized, placebo-controlled, double-blind trial to investigate the effectiveness of imiquimod 5% cream in VIN. It was observed that in 81% of cases treatment with imiquimod led to a reduction of lesion size, in comparison with 0% in the placebogroup ($p<0.001$).[16] In addition to this, the reduction of lesion size was correlated with partial normalization of the numbers of immunocompetent cells. Furthermore, it was also shown that arousal of HPV16-specific type 1 cellular immunity by induction of local inflammation by imiquimod was involved in regression of HPV related VIN lesions.[17]

In the current study we are further exploring the local immune responses in VIN. Using earlier produced gene expression profiles of HPV related VIN tissues and healthy vulvar control tissues[18], here we analyzed the expression of cytokines and cytokine receptors. In addition, the expression of specific cytokines (*CCL20*, *CCL21*, *CCL22*, *CXCL10*, *IL12*), the Treg cell marker *FOXP3* and the inflammation-inducer *PPAR*γ was analyzed in 14 new VIN patients. From each of these 14 patients one affected (HPV-positive VIN lesion) and one non-affected (HPV-negative) vulvar tissue was obtained for this study. Subsequently, data were related to the presence or absence of immunocompetent cells (DCs and T-cells) in affected and non-affected vulvar tissues.

MATERIALS & METHODS

Patient samples

All patients (n = 14) were histologically and clinically diagnosed with high-grade VIN. From these 14 women we collected a 4 mm punch biopsy of the affected vulvar skin (HPV-positive VIN lesion) and a 4 mm punch biopsy from contra-lateral non-affected, healthy vulvar skin (HPV-negative). These two biopsies were taken at the same time and were directly frozen in liquid nitrogen and stored at -80°C until further analysis. All samples (affected and non-affected) were reviewed by a pathologist (PCE) for histological diagnosis and were analyzed for the presence of HPV DNA by using a standard GP5$^+$/6$^+$ PCR enzyme immunoassay (EIA) followed by reverse line blot analysis, as described previously.[19] The medical Ethical Committees approved our study design and all women voluntarily gave written informed consent.[16]

Microarray data analysis

Microarray data were obtained from an earlier study, where we compared HPV-related VIN to healthy vulvar skin obtained from women visiting a medical center for plastic cosmetic surgery for reduction of the labia minora.[18] To identify differences in cytokine and cytokine receptor expression between VIN and control tissue, genes involved in the *cytokine and cytokinereceptor interaction* pathway (KEGG PATHWAY database: http://www.genome.jp/dbget-bin/www_bget?path:hsa04060) were selected and used as a starting point for our current investigations. Raw and normalized microarray data have been deposited in the GEO repository at NCBI under accession GSE5563.

Immunohistochemical staining

Immunohistochemical staining was performed for the following markers: CD1a, classic marker for Langerhans Cells; CD207, marker for immature Langerhans Cells expressing Langerin; CD208, marker for mature DCs;

CD94, marker for NK cells; CD4, marker for T-helper cells; CD8, marker for cytotoxic T-cells, and CD25/HLA-DR, marker for Treg cells. For plasmacytoid DCs, characterized by the presence of CD123 and absence of CD11c, both markers were used. Stainings and light microscopic evaluation of the obtained data were performed as described earlier.[14]

Quantitative real-time RT-PCR

Total RNA was isolated from tissue samples using Trizol (Invitrogen, Life Technologies, Philadelphia, PA, USA) and quality and quantity was assessed on the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). RNA was considered of sufficient quality with an RNA Integrity Number (RIN-value) of 7.5 or higher. Accordingly, cDNA was generated from 1 µg total RNA from 18 samples (from each of 9 patients, one affected and one non-affected vulvar tissue was obtained) using T7 oligo d(T) primers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen) according to the Affymetrix protocol for first strand cDNA synthesis (Affymetrix, Santa Clara, CA, USA). Real-time PCR (RT-PCR) was performed in duplicate using the Opticon I (Applied Biosystems) and SYBR Green I™ (Applied Biosystems, Foster City, CA, USA). 10 ng of the cDNA samples were amplified with 0.5 µM of primer pairs specific for the genes, in a 25 µl reaction with 12.5 µl SYBR Green PCR master mix (Applied Biosystems). The housekeeping gene β-actin was used for normalization and all PCR primers were designed to be intron spanning (Table 1). PCR reactions were performed as follows: 38 cycles of denaturation at 95^{0}C (15 seconds), annealing at 59-62^{0}C (30 seconds), and extension at 72^{0}C.

The comparative C_T method (Applied Biosystems) was used to determine relative quantitation of gene expression for each gene compared with the β-actin control. The difference in cycle time ΔC_T, was determined as the difference between the tested gene and the reference housekeeping gene, β-actin. $\Delta\Delta C_T$ was then found by obtaining the difference between each sample compared to the mean of the expression values of the control group. The relative fold change was calculated as $FC=2^{-\Delta\Delta CT}$.

2.2

Table 1. Primers used for real-time PCR.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
PPARγ	TTCAGAAATGCCTTGCAGTG	CCAACAGCTTCTCCTTCTCG
CCL20	TTTATTGTGGGCTTCACACG	GATTTGCGCACACAGACAAC
CCL21	TATCCTGGTTCTGGCCTTTG	CAGCCTAAGCTTGGTTCCTG
CCL22	CGCGTGGTGAAACACTTCTA	CGGCACAGATCTCCTTATCC
CXCL10	CCACGTGTTGAGATCATTGC	TTCTTGATGGCCTTCGATTC
IL12A	AAGGAGGCGAGGTTCTAAGC	TTCTTGATGGCCTTCGATTC
FOXP3	TCCCAGAGTTCCTCCACAAC	ATTGAGTGTCCGCTGCTTCT

Statistics

For the selected cytokine and cytokine receptor mRNA levels, the Mann-Whitney test was used for evaluation of differences in signal intensity between the normalized microarray data (VIN versus healthy controls). A p-value <0.05 was considered statistically significant. For the immunocompetent cell counts and quantitative real time RT-PCR, Wilcoxon Signed Ranked Test was used to calculate significant differences between affected and one non-affected vulvar tissues. A p-value <0.05 was considered statistically significant.

RESULTS

Patient characteristics

Biopsies specimens were obtained from 14 women, aged 32 to 58 years (median age: 43 years). From each of these 14 patients one affected (HPV-positive VIN lesion) and one contra-lateral non-affected (HPV-negative) vulvar tissue was obtained. Patient characteristics of those 14 women are described in Table 2.

Expression of cytokines and cytokine receptors in VIN (microarray data)

In our earlier work[18] we observed in VIN tissue a 11-fold decreased expression of PPARγ, a receptor molecule which has been implicated in maturation of dendritic cells.[20] Here, we have reanalyzed these microarray data to review differential expression of genes involved in initiating and maintaining an immune response. From the KEGG database we have obtained a comprehensive scheme (http://www.genome.jp/dbget-bin/www_bget?path:hsa04060) on "cytokine-cytokine receptor interaction". In Figure 1 this scheme was used as a background for our own microarray data. In Figure 1 all significantly up- (red) or down-regulated (green) "cytokine-cytokine receptor-interaction" genes in VIN as compared to control tissue are shown.

Up-regulation of the following genes was observed in VIN lesions: Chemokines + receptors: *IL8*, *CXCL10*, *CCL20*, *CCL22* and *CCR7*; Hematopoietins + receptors: *IL2RG*; PDGF-family + receptors: *VEGF*, *IFNGR1* and *IL28RA*; TNF-family + receptors:

Table 2. Patient characteristics.

Patient	Age	Histology	Smoking	HPV type in VIN lesion	HPV type in non-affected tissue	Immuno-staining	RT-PCR
1	34	VIN3	Yes	Neg	Neg	Yes	Yes
2	47	VIN3	Yes	16	Neg	Yes	Yes
3	41	VIN3	Yes	18	Neg	Yes	Yes
4	40	VIN3	Yes	16	Neg	Yes	Yes
5	53	VIN3	Yes	16	Neg	Yes	Yes
6	46	VIN3	Yes	16	Neg	Yes	Yes
7	32	VIN3	Yes	16	Neg	Yes	Yes
8	39	VIN3	Yes	16	Neg	Yes	Yes
9	48	VIN3	Yes	16	Neg	Yes	Yes
10	45	VIN3	Yes	16	Neg	Yes	No
11	45	VIN3	Yes	16	Neg	Yes	No
12	34	VIN3	Yes	33	Neg	Yes	No
13	39	VIN3	No	16	Neg	Yes	No
14	58	VIN3	Yes	16	Neg	Yes	No

TNFSF10, *TNFRSF10A*, *TNFRSF10B*, *TNFRSF25* and *TNFRSF7*; TGFbeta-family + receptors: *TGFA*, *INHBA*, *ACVR1B* and *BMP*; IL17-family + receptors: *IL17RB*; and IL1-family + receptors: *IL1F9* and *IL1F5*.

Down-regulation of the following genes was observed in VIN lesions: Chemokines + receptors: *IL8RB*, *CXCL12*, *CCL21* and *CCL14*; Hematopoietins + receptors: *IL11RA*, *LIFR*, *LEPR* and *IL13RA* ; PDGF-family + receptors: *PDGFA*, *PDGFB*, *PDGFC*, *PDGFD*, *PDGFRB*, *VEGFB*, *VEGFC*, *KDR*, *EGFR*, *CSF1*, *CSF1R*, *KITLG*, *KIT*, *IFNGR2* and *IL20RA*; TNF-family + receptors: *TNFSF12* and *TNFSF13*; TGFbeta-family + receptors: *TGFB1*, *TGFB4*, *INHBB*, *TGFBR3*, *ACVR2* and *BMPR2*; IL17-family + receptors: *IL17D*, *IL17RC* and *IL17RD* ; and IL1-family + receptors: *IL1R2*.

Detection of immunocompetent cells

The above observations showed that during a persistent HPV infection, several immunomodulatory proteins are regulated, suggesting an ongoing immune response. In order to investigate this further, we have measured the presence of immunocompetent cells in affected (HPV-positive VIN lesion) and non-affected (HPV-negative) vulvar tissue obtained from 14 patients. Overall, immunocompetent cells could easily be detected by immunohistochemical staining in affected and non-affected vulvar tissues (Figure 2) and in general, more cells were present in the dermis than in the epidermis.

CYTOKINE - CYTOKINERECEPTOR INTERACTION

Chemokines

CXC subfamily

CXCL1 CXCL2 CXCL3 CXCL5 CXCL7 CXCL6 IL8 → IL8RB, IL8RA
CXCL9 CXCL10 CXCL11 → CXCR3
CXCL12 → CXCR4
CXCL13 → BLR1
CXCL16 → CXCR6
CXCL15 → ?
CXCL4 → ?
CXCL14 → ?

C subfamily

XCL1 XCL2 → XCR1

CX3C subfamily

CX3CL1 → CX3CR1

CC subfamily

CCL1 → CCR8
CCL20 → CCR6
CCL25 → CCR9
CCL17 CCL22 → CCR4
CCL19 CCL21 → CCR7
CCL2 CCL12 → CCR2
CCL4 CCL3 → CCR5
CCL13 CCL7 CCL5 CCL14 CCL16 CCL23 → CCR1
CCL15 CCL8 CCL11 CCL24 CCL26 → CCR3
CCL28 CCL27 → GPR2
CCL6 → ?
CCL9 → ?
CCL18 → ?

Hematopoietins

gp130 (IL6ST) shared

IL6 → IL6R α, IL6ST gp130
IL11 → IL11RA α, IL6ST gp130
human
OSM → LIFR β, IL6ST gp130
human / murine
OSM → OSMR β, IL6ST gp130
LIF → LIFR β, IL6ST gp130
CNTF → CNTFR α, LIFR β, IL6ST gp130
BSF3 → CNTFR α, LIFR β, IL6ST gp130
CTF1 → ?, LIFR β, IL6ST gp130
CSF3 → CSF3R
LEP → LEPR

IL13RA1 shared

IL4 → IL4R α, IL13RA α
IL13 → IL4R α, IL13RA α

IL12RB1 shared

IL12 → IL12RB1 β, IL12RB2 β
IL23A → IL23R, IL12RB1 β

IL-3RB (CSF2RB) shared

CSF2 → CSF2RA α, CSF2RB β
IL3 → IL3RA α, CSF2RB β
IL5 → IL5RA α, CSF2RB β

IL2RG shared

IL2 → IL2RA α, IL2RB β, IL2RG γ; IL2RB β, IL2RG γ
IL4 → IL4R α, IL2RG γ
IL7 → IL7R α, IL2RG γ
IL9 → IL9R α, IL2RG γ
IL15 → IL15RA α, IL2RB β, IL2RG γ
IL21 → IL21R α, IL2RG γ
TSLP → IL7R α, TSLPR α

Single chain

EPO → EPOR
GH1 GH2 → GHR
PRL → PRLR
TPO → MPL

PDGF Family

Receptor tyrosine kinase

PDGFC PDGFA PDGFB PDGFD → PDGFRA α, PDGFRA α, PDGFRB β, PDGFRB β
VEGFB VEGF VEGFE VEGFC → FLT1, KDR, FLT4
HGF → MET
EGF → EGFR
CSF1 → CSF1R
KITLG → KIT
FLT3LG → FLT3

Interferon Family

IFNA IFNB1 IFNW1 IFNK IFNT1 → IFNAR1 α, IFNAR2 β
IFNG → IFNGR1 α, IFNGR2 β

IL-10 Family

IL10 → IL10RA, IL10RB
IL19 IL20 IL24 → IL20RA, IL20RB; IL22RA1, IL22RB
IL22 → IL22RA1, IL10RB; IL22RA2, ?
IL28A IL28B IL29 → IL28RA, IL10RB
IL26 → ?

TNF Family

TNFR

TNFSF15 TNFSF10 → SF10A, SF10B, SF10C, SF10D, SF11B
TNFSF11 → SF11A
SF25
TNFSF12 → SF12A
SF21
SF16
TNF LTA LTB TNFSF14 → SF1B, SF1A, LTBR, SF14, SF6B
TNFSF6 → SF6
TNFSF5 → SF5
TNFSF7 → SF7
TNFSF8 → SF8
TNFSF9 → SF9
TNFSF4 → SF4
TNFSF18 → SF18
TNFSF13 TNFSF13B → SF17, SF13B, SF13C
EDA-A1 EDA → EDAR
EDA-A2 EDA → XEDAR
SF19
SF19L

TGF-β Family

TGFB1 TGFB2 TGFB3 → TGFBR2 Type II, TGFBR1 Type I
INHBA INHBB INHBC INHBE → ACVR2 Type II, ACVR1 Type I; ACVR2 Type II, ACVR1B Type I; ACVR2B Type II, ACVR1 Type I; ACVR2B Type II, ACVR1B Type I
AMH → AMHR2 Type II, ACVR1? Type I
BMP2 → BMPR2 Type II, ACVR1 Type I; BMPR2 Type II, BMPR1B Type I; BMPR2 Type II, BMPR1A Type I
BMP7 → ACVR2 Type II, BMPR1B Type I
GDF5 → ACVR2 Type II, BMPR1A Type I

IL-17 Family

IL17A → IL17R
IL17B IL17E → IL17RB

IL-1 Family

IL1A IL1B → IL1R1, IL1RAP; IL1R2, IL1RAP (No signal transduction)
IL18 → IL18R1, IL18RAP

DCs: For (immature) Langerhans Cells ($CD1A^+$ and $CD207^+$) higher numbers were observed in the epidermis, and no significant differences were observed between affected and non-affected vulvar tissues. For mature ($CD208^+$) and plasmacytoid DCs ($CD123^+/CD11c^-$) the situation was almost opposite: higher numbers of these DCs were observed in the dermis and a significant increase in cell numbers was observed in affected as compared to non affected vulvar tissues for mature DCs. For plasmacytoid DCs the difference in cell-counts in the dermis did not reach significance between affected and non-affected vulvar tissues ($p = 0.088$).

NK Cells: NK cells ($CD94^+$) were present in approximately similar numbers in epidermis and dermis and no significant differences were observed in NK cell-counts between affected and non-affected vulvar tissues.

T Cells: Compared to the epidermis, T-cells were enriched in the dermis. Furthermore, the most abundant T-cell type was the T-helper cell ($CD4^+$), which was found in significantly higher numbers in the dermis of VIN as compared to non-affected vulvar tissue. The distribution of cytotoxic T-cells ($CD8^+$) was comparable to that of T-helper cells: Cytotoxic T-cells were also enriched in the dermis and significantly higher numbers were observed in VIN lesions as compared to non-affected tissues. For Treg cells ($CD25^+/HLA\text{-}DR^-$) low numbers were found in both epidermis and dermis. The Treg cell-numbers in the dermis were higher than in the epidermis, but no differences were observed between VIN lesions and non-affected vulvar tissues.

Real-time PCR

Based on the finding that $CD208^+$, $CD8^+$ and $CD4^+$ cells were more abundant in the dermis of affected vulvar skin, and based on reports in literature [21-23] a number of specific cytokines (*CCL20*, *CXCL10*, *IL12A*, *CCL22*, *CCL21*), the Treg cell marker *FOXP3* and the inflammation-inducer *PPARγ* were chosen for quantitative real-time RT-PCR.

RNA integrity was guaranteed by only using samples with an RNA Integrity Number (RIN) of 7.5 or higher. Consequently, 9 of 14 patients were included. We performed real-time PCR analysis in VIN lesions and their corresponding non-affected vulvar tissues (Figure 3 and Table 3). Analysis of these data by comparing the two groups (affected versus non-affected vulvar tissues) showed that only *PPARγ* was significantly down regulated in VIN lesions ($p=0.001$). The other genes were clearly not regulated significantly in VIN (*FOXP3*, *CXCL10* and *IL12A*) or significance was

◁ **Figure 1.** Cytokine and cytokinereceptor expression in VIN lesions. From the KEGG database (www.genome.jp/KEGG/pathway.html) the scheme on "cytokine-cytokine receptor interaction" was used to overlay our significantly differentially expressed genes between VIN and control. The Mann-Whitney test was used for evaluation of differences in signal intensity of the normalised microarray data (VIN versus healthy controls). A p-value of 0.05 was considered statistically significant. Red boxes represent genes with a significantly higher expression in VIN compared to controls; green boxes represent genes with a significantly lower expression in VIN compared to controls.

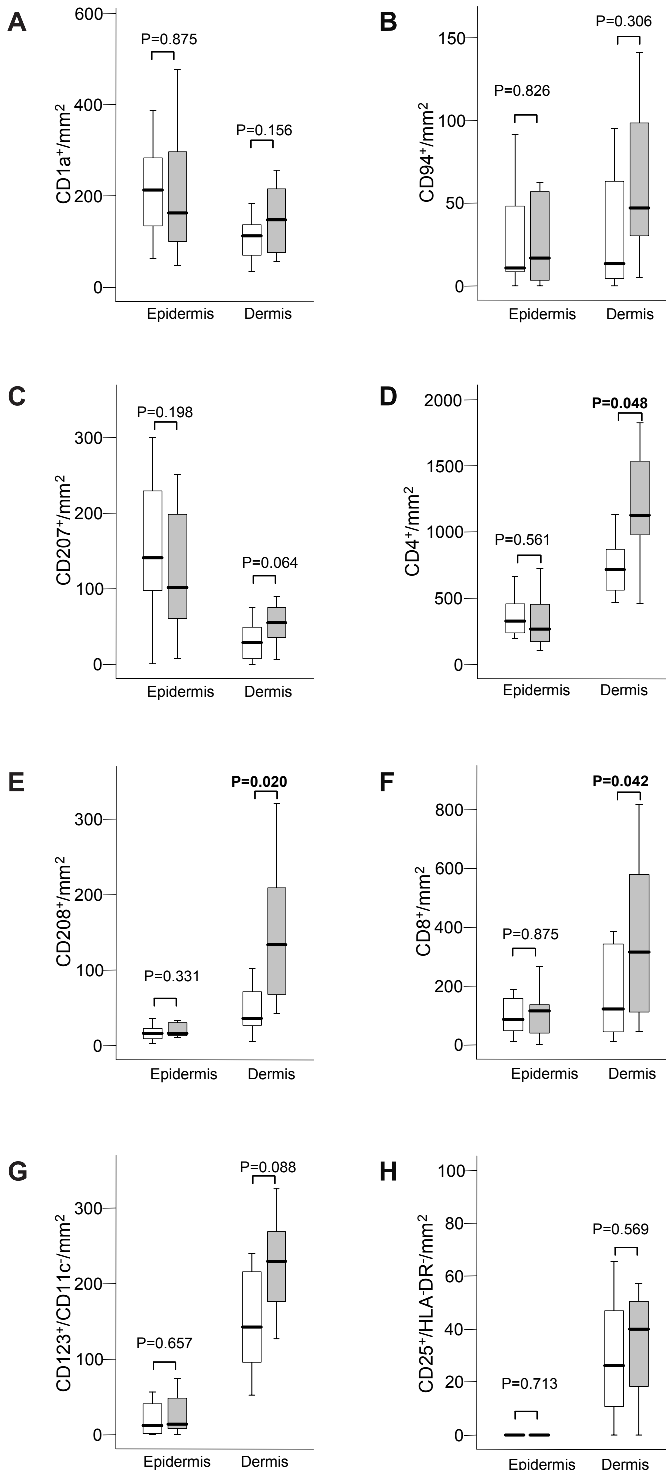

Figure 2. Immunocompetent cells in VIN and control tissue. Immunohistochemical staining was performed for different markers indicated at the Y-axis of each figure. **A:** Langerhans cells (CD1a+); **B:** Natural killer cells (CD94+); **C:** Immature Langerhans cells (CD207+); **D:** T-helper cells (CD4+); **E:** Mature dendritic cells (CD208+); **F:** Cytotoxic T-cells (CD8+); **G:** Plasmacytoid dendritic cells (CD123+/CD11c-); **H:** Regulatory T-cells (CD25+/HLA-DR). Positive cells were counted in the epidermis as well as in the dermis and were represented per mm^2. Median values are indicated by horizontal lines in the box plots. Wilcoxon Signed Ranked Test was used to calculate significances. White boxes represents control tissue and grey boxes VIN samples from the same patients (n=14).

not reached (*CCL20*, p = 0.115; *CCL21*, p = 0.151; and *CCL22,* p = 0.084). In Table 3 differences per patient are being evaluated. Interestingly in Figure 1, showing microarray data from our earlier study [18], *CCL20* and *CCL22* are indeed significantly upregulated in VIN lesions while *CCL21* is significantly down-regulated in VIN lesions. This is in accordance with the observed trends towards significance in our RT-PCR experiment (Figure 3 and Table 3).

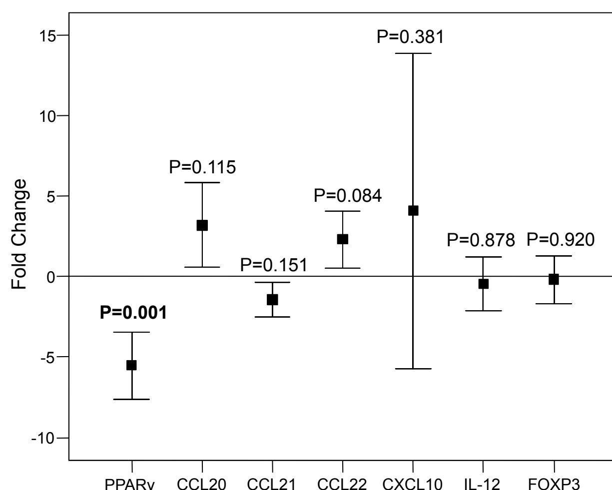

Figure 3. Real-time RT-PCR results. On the X-axis the different investigated genes are indicated. On the Y-axis the ratio between the measured expression level in VIN lesions versus control tissues from the same patients is indicated. All values have been corrected for the expression of the household gene β-Actin. Median values are indicated by horizontal lines in the box plots. Wilcoxon Signed Ranked Test is used to calculate significances.

Table 3. Quantitative real-time RT-PCR results per patient.

	Patient number											
Gene name	1	2	3	4	5	6	7	8	9	Up[a]	Down[a]	p-value[b]
PPARγ	-11,1	-13	-1,1	-5,8	-3,4	-3,4	-8,3	-2	-11,9	0/9	9/9	0,001
CCL20	7,8	12,3	3,4	9,4	1,1	-6,2	1,4	18	-4,2	7/9	2/9	0,115
CCL21	-5,3	-4,1	-1	-2,2	-1	1,2	1,2	1,8	-2,1	3/9	6/9	0,151
CCL22	3,1	4,1	-4,5	3,5	6,4	1,1	5,6	2,5	-1,4	7/9	2/9	0,084
CXCL10	-4,3	7,9	1,7	1,2	-1,1	-1,3	1,9	2	1,2	6/9	3/9	0,381
IL12	-1,5	-1,1	1,6	-5,1	2,5	1,8	-1,7	-1,3	1,8	4/9	5/9	0,878
FOXP3	-1,5	-2	-2,2	-2,2	3	1,5	-1,3	3	1,6	4/9	5/9	0,92

Data are expressed as Fold Change differences, calculated by dividing the VIN expression value by the control expression value.
[a] Describes in how many patients the gene is upregulated or downregulated
[b] Wilcoxon Signed Ranked Test is used to calculate significances between all VIN samples in comparison with all control samples.

DISCUSSION

Forty percent of women are, during their lifetime, infected by any of the high-risk HPV types, and although the majority of HPV-infections are cleared from the system, the HPV virus sometimes presents a problem for the immune system. HPV exclusively infects and multiplies in keratinocytes, which are located distant from lymph nodes. Furthermore, keratinocytes have a short lifespan and because of this, the virus does not need to lyse the cell, which avoids inflammation as a potent trigger for the first-line innate immune response. Also, HPV downregulates the expression of *TLR-9*, which is one of the HPV binding receptors in DCs, and HPV reduces the production of interferon, which plays an important role in the adaptive immune response.[24]

The current manuscript examines the immune response in HPV-positive VIN lesions in comparison to HPV-negative, non-affected vulvar tissues in the same patient. More particularly, the expression of different cytokines in relation to immunocompetent cell numbers into the affected region was assessed. This in order to obtain better understanding of the local immune response in patients with VIN.

The innate immune response (Figure 4)

CXCL12 is an important chemoatttracting agent, which is produced by stromal cells in response to inflamation.[25] In our microarray analysis, the expression of *CXCL12* was significantly downregulated, suggesting that the process of migration of DCs into the affected area is significantly disturbed. To investigate this further, we studied the presence of antigen presenting DCs in the dermis and epidermis of HPV-related VIN in comparison to control vulvar tissues from the same patient. Overall, we measured

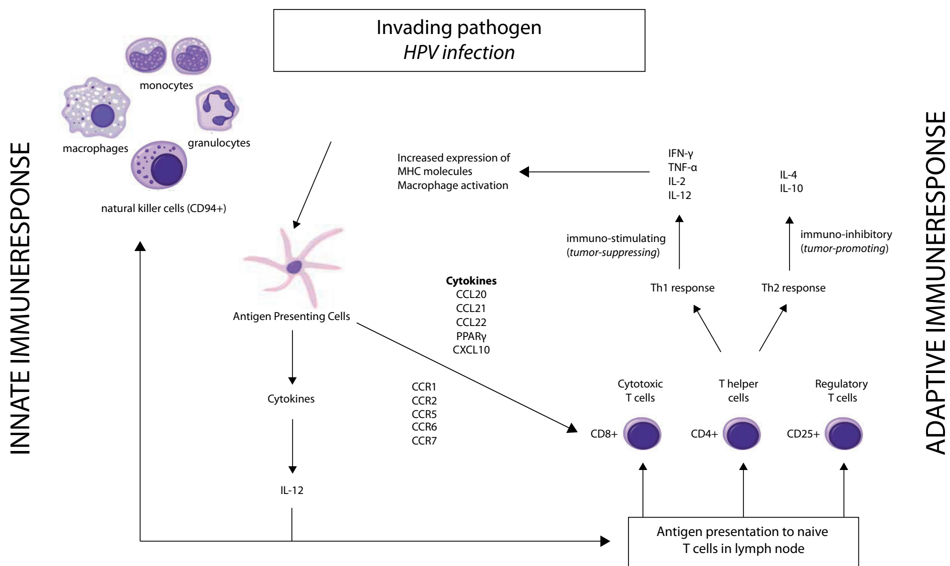

Figure 4. Cells and cytokines involved in the innate and adaptive immune response.

higher levels of DCs in the dermis as compared to the epidermis. Furthermore, in VIN affected tissue, the cell numbers seemed to decrease in the epidermis while there was a slight increase in DCs in the dermis (Figure 2, $CD208^{+}$, p=0.020). These observations were in good accordance with earlier findings of our group[14] and suggest that after recognition of viral antigens, DCs are stimulated to move towards the dermis, while new DCs are hampered to fill up the epidermal niche.

Under influence of different signals, DCs will mature and migrate towards local lymphoid tissues to present antigens to naive T cells (Figure 4). This trafficking process has been described as resulting from a switch in chemokine receptor expression at the DC cell surface.[26] Migration of DCs towards the draining lymph node is stimulated by on the one hand down-regulation of the receptors of inflammatory chemokines (*CCR1*, *CCR2*, *CCR5* and *CCR6*) and on the other hand up-regulation of the receptors for lymphoid chemokines (*CCR7*). In VIN a number of observations suggests that DC-maturation is taking place. Firstly, upon measuring $CD208^{+}$ cells (CD208 is a marker for mature DCs), we found significantly increased numbers of mature DCs in the dermis (Figure 2). Secondly, our microarray as well as quantitative real-time RT-PCR data showed a strong and significant down-regulation of *PPAR*γ, which has been implicated in DC functional maturation. More specifically, Klotz et al. studied *PPAR*γ-deficient DCs and showed that absence of this gene leads to increased DC immunogenicity.[20] And last, we found a significant increase in *CCR7* expression, which is expressed in mature DCs. These findings indicate that in VIN, mature DCs are present at the affected site and ready to migrate towards the lymph node. However, this migration process seems to be disturbed, since one of the most important ligands of the *CCR7* receptor, namely *CCL21*, is significantly downregulated. Due to this lack of accurate chemokine signaling, mature DCs seem to be bottled up in the dermis (Figure 2, CD208+, p=0.020).

In conclusion, it seems that most mature DCs do not receive the proper chemokine signal for migration and will stay in the skin, not able to present the viral antigen to naive T cells in the lymph node. Perchance this may be one of the reasons of an inaccurate initiation of the adaptive immune response.

The adaptive immune response (Figure 4)

Homing of effector T cells to sites of infection is dependent on different chemokines. For example *CXCL10* is known to be the chemotactic and proliferation factor for certain T-helper cells (Th1) and chemotatic for DCs.[27] We found an up-regulation of *CXCL10* and in line with this observation we found higher numbers of $CD4^{+}$ (T-helper cells) and $CD8^{+}$ cells (cytotoxic T-cells) in the dermis of VIN. Furthermore a significant correlation (Pearson correlation, p=0.001) was observed between numbers of T-helper cells and the expression of *CXCL10* (Table S1, supplementary data (http://www.erasmusmc.nl/47393/1584119/1603959/Santegoets)). Gul et al. examined low and high grade VIN and found only a 3-fold increase in T-helper cells and cytotoxic T-cells, which is in accordance to our results.[13] Interestingly, Bourgault et al. could show that a successful clearance of high grade VIN was accompanied by a strong

epidermal and dermal T-helper and cytotoxic T-cell infiltration, something which was not observed in the current investigations.[12] Combining our current data with data from literature, it seems that although a T-cell response is observed in persistent VIN, a much stronger response is required in order to clear the infection.

Investigations into the working mechanism of the immuno-modifying agent imiquimod in the treatment of VIN, also supports this hypothesis: Todd et al.[28] showed that application of imiquimod increased the magnitude of the cytotoxic T-cell response and when imiquimod was used in the treatment of actinic keratosis, it stimulates a cutaneous immune response characterized by increases in activated DCs and T-helper and cytotoxic T cells.[29] In addition upon reviewing imiquimod responding patients, van Seters also observed that imiquimod treatment resulted in influx of cytotoxic T-cells into the epidermis of VIN lesions.[16]

T-cell activation results in the secretion of different cytokines that help and regulate other immunocompetent cells. The pattern of cytokine expression is dependent on the pathway that is activated. In general, two main responses are initiated by T-helper cells: a Th2 type response, in which IL-4 and IL-10 are the key players; or a Th1 type response, which is important to fight viral infections (Figure 4). Van Poelgeest et al. could show that the Th1 type response plays an important role in the protection against progressive HPV related VIN by recognizing the HPV early antigens E2, E6 and E7.[17] One of the produced cytokines during a Th1 type response is IFN-γ, which is crucial for an effective innate as well as adaptive immune response. IFN-γ is known to act as a potent effector in limiting viral replication and increasing resistance to infection. In the current study, we did not observe increased expression of IFN-γ mRNA in VIN tissues. This finding is in accordance with observations in Cervical Intraepithelial Neoplasia (CIN) where high-grade CIN is associated with decreased expression of the Th1 type cytokines, tumor necrosis factor-α and IFN-γ.[30-32] In the absence of a sufficient Th1 type response, cytotoxic T-cells will not migrate and differentiate, which will result in reduced anti-viral and anti-tumor immunity.

In summary, analysis of the immune response during HPV related VIN revealed that an ineffective innate immune response may be an important factor causing a significantly reduced adaptive immune response. Stimulation of innate as well as adaptive immunity may therefore represent an important instrument to treat persistent HPV infections, which may otherwise develop further in neoplasia.

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2.3

DIFFERENT DNA DAMAGE AND CELL CYCLE CHECKPOINT CONTROL IN LOW- AND HIGH-RISK HUMAN PAPILLOMAVIRUS INFECTIONS OF THE VULVA

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ABSTRACT

Human papillomavirus (HPV) infections may result in benign hyperplasia, caused by low-risk HPV types, or (pre)malignant lesions caused by high-risk HPV types. The molecular basis of this difference in malignant potential is not completely understood. Here, we performed gene profiling of different HPV infected vulvar tissues (condylomata acuminata (n=5), usual type Vulvar Intraepithelial Neoplasia (uVIN) (n=9)) and control samples (n=14) using Affymetrix Human U133A plus 2 GeneChips. Data were analysed using OmniViz®, Partek® and Ingenuity® Software. Results were validated by realtime RT-PCR and immunostaining.

Although similarities were observed between gene expression profiles of low- and high-risk HPV infected tissues (for example absence of Estrogen Receptor in condylomata and uVIN), high-risk HPV infected tissues showed more proliferation and displayed more DNA damage than tissues infected with low-risk HPV. These observations were confirmed by differential regulation of cell cycle checkpoints and by increased expression of DNA damage-biomarkers p53 and γH2AX. Furthermore, *FANCA*, *FANCD2*, *BRCA1* and *RAD51*, keyplayers in the DNA damage response, were significantly upregulated ($P<0.05$). In addition we compared our results with publicly available gene expression profiles of various other HPV-induced cancers (vulva, cervix and head and neck). This showed $p16^{INK4a}$ was the most significant marker to detect a high-risk HPV infection, but no other markers could be found.

In conclusion, this study provides insight into the molecular basis of low- and high-risk HPV infections and indicates two main pathways (cell cycle and DNA damage response) that are much stronger affected by high-risk HPV as compared to low-risk HPV.

INTRODUCTION

Worldwide, human papillomavirus (HPV) is the most common sexually transmitted infection, with an 80% life-time infection risk.[1] Fortunately, the majority of these HPV infections (~90%) are cleared within one to two years, without further consequences for the host.[2] Persistent infections, however, are a well-established risk factor for a large spectrum of epithelial lesions, ranging from benign hyperplasia, caused by low-risk HPV types, to (pre)malignant lesions caused by high-risk HPV types.

The best known high-risk HPV related disorder is the second most common cancer in women, namely cervical cancer, with 500.000 new cases each year worldwide resulting in 250.000 deaths every year.[3] Persistent HPV infections have also been associated with other anogenital squamous cell carcinomas, including vulvar, vaginal, anal and penile cancers and their precursors. Furthermore, recent epidemiological, molecular and clinical evidence indicate that high-risk HPV (especially HPV type 16) accounts for the development of approximately 20-30% of squamous cell carcinomas of the head-and-neck.[4-5]

The molecular basis of the difference in malignant potential between low- and high-risk HPV infections is not completely understood. Most likely this is caused by differences in the ability of oncoproteins E6 and E7 to induce transformation of cells.[6] To expand our knowledge of the oncogenic processes induced by persistent high-risk HPV infection, we made a direct comparison between the cellular effects of low- and high-risk HPV infection in vulvar tissue.

Infections with low-risk HPV types, for example 6 and 11, can result in benign vulvar disorders like condylomata acuminata. Condylomata are the most common viral sexually transmitted infection in the world. National cumulative prevalence in the United States is estimated to be 6% of the sexually active population aged 18-59 years.[7-8] Condylomata are often multifocal and can lead to a variety of symptoms, including anogenital pruritus, burning, vaginal discharge, or bleeding. There are a variety of treatment options, including antiproliferative, immunomodulatory and destructive/surgical therapies. However, most condylomata are self-limiting and spontaneously regress.

In contrast to an infection with low-risk HPV, persistent infections with high-risk HPV types, such as 16, 18 and 33, can cause high grade intraepithelial neoplasia. The persistence of these high-grade HPV infections may be due to diminished responsiveness of the adaptive immune system.[9] Integration of the HPV-DNA into the host's genome is considered as one of the key events in the pathogenesis of HPV related cancers.[10] It is found in many high-grade lesions and cancers, while it is rarely observed in low-grade lesions. This phenomenon has mainly been studied in cervical carcinogenesis, where the proportion of samples with integration of HPV 16/18 increases with the severity of cervical lesions.[11-13] Few data, however, are available on HPV integration in vulvar lesions.[14-15]

Vulvar neoplasia caused by a persistent HPV infection is known as usual type Vulvar Intraepithelial Neoplasia (uVIN). This disorder can eventually develop into invasive

squamous cell carcinoma of the vulva. The percentage of women with VIN that develop cancer is difficult to determine, because most patients are treated effectively to prevent invasive disease. A review about 3,322 patients with VIN showed that 8/88 (9%) untreated patients progressed to vulvar cancer within 1-8 years.[16]

In the current study, gene expression profiling was performed on HPV related vulvar disorders (condylomata and uVIN). It was observed that low and high-risk HPV types differ in their ability to control DNA damage and cell cycle checkpoints. Furthermore, we compared our results with publicly available gene expression profiles in various other HPV-induced cancers (vulva, cervix and head and neck). This showed $p16^{INK4a}$ was the most significant marker to detect a high-risk HPV infection, but no other markers could be found.

2.3

MATERIAL AND METHODS

Samples

Biopsies from normal vulvar tissue were obtained from women undergoing elective vulvar cosmetic surgery in a private medical center. Condylomata were collected from women attending the outpatient's clinic for sexually transmitted diseases at the department of Dermatology and Venereology at the Erasmus University Medical Center, Rotterdam. All uVIN samples were collected at the start of a randomized clinical trial investigating the effectiveness of 5% imiquimod cream.[17] All women were premenopausal (median age controls: 40 years (range 17-54); condylomata: 26 years (range 19-41); uVIN: 39 years (range 33-48) and provided informed consent. All diagnoses were confirmed by a pathologist (PCE). HPV testing was performed on all uVIN and control lesions by using the GP5+/6+ PCR enzyme immunoassay, as described previously.[18] No HPV was detected in the control samples, while high-risk HPV was detected in uVIN samples (HPV16 (n=5), HPV33 (n=3) and HPV of unknown type (n=1)). HPV testing in condylomata was performed by using the INNO-LiPA HPV Genotyping test (Innogenetics, Ghent, Belgium). In all condylomata samples low-risk HPV type 6 was detected. In addition, 2 samples contained more HPV types (HPV type 11, 35 en 52). Tissues were frozen in liquid nitrogen and stored at -80°C until processing. Cryosections of 10 μm were obtained and the first and tenth section in a series of 10 was stained to assess tissue morphology. The remaining sections were used to isolate total RNA using Trizol (Invitrogen, Life Technologies, Philadelphia, PA, USA) and quality and quantity was assessed on the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA).

Microarray and RT-PCR experiments were performed using total RNA of 14 control samples, 5 condylomata and 9 uVIN samples. In order to verify reproducibility for the microarray experiment we arrayed 4 control and 2 condylomata samples twice.

For immunohistochemistry we selected condylomata (n=10), uVIN (n=23) and control samples (n=20). These samples were obtained between 1995 and 2008 from the pathology archives of the Erasmus Medical Center, Rotterdam, the Netherlands.

Microarray and microarray analysis

Affymetrix U133plus2 GeneChips were used to obtain gene expression profiles. Staining, washing and scanning procedures were carried out as described by the manufacturer (Affymetrix, Santa Clara, CA, USA). Raw (.CEL files) and normalized microarray data have been deposited in the GEO repository at NCBI under accession number GSE5563.

To relate the findings of our study to studies of others, we searched in the two most commonly used databases for microarray data, namely ArrayExpress and NCBI's Gene Expression Omnibus (GEO), for published or unpublished data on HPV related disorders. The search keywords were HPV, intraepithelial neoplasia, condylomata, VIN, VaIN, AIN, CIN, head&neck, cancer and dysplasia. To achieve complete coverage of the literature and limit report bias, we evaluated all published articles cited. Only studies using the Affymetrix GeneChip Human Genome U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) were included for further analysis.

To examine the quality of the various arrays, measured intensity values were analyzed using the AffyQC algoritm of the GeneChip Operating Software (Affymetrix, Santa Clara, CA, USA). Subsequently, raw intensities of each chip were log2 transformed and normalized using quantile normalization using R or RMA express. Batch effects were removed by using Partek® software (Partek Inc., St. Louis, MO, USA). Data analysis was carried out using OmniViz software version 3.8 (OmniViz, Maynard, MA, USA) and Partek® software (Partek Inc., St. Louis, MO, USA).

Differentially expressed genes with respect to HPV exposure were selected by controlling the false discovery rate (FDR; by ANOVA for each gene separately). A FDR (adapted for multiple testing) less than 1% was considered statistically significant. Using OmniViz software hierarchical clustering of differentially expressed genes and/or samples was performed.

Ontological analysis, using Ingenuity Pathway software (Ingenuity® Systems, www.ingenuity.com), was employed to assess the functional relevance of the observed differences in gene expression profiles.

Immunohistochemistry

Serial tissue sections (4 μm thick) of formalin-fixed and paraffin-embedded blocks were obtained and every first and last section was hematotoxylin and eosin-stained in order to confirm diagnosis by a pathologist (PCE).

Prior to incubation with the primary antibodies for Ki-67 (Dako, Glostrup, Denmark), ERα (Thermoscientific, Fremont, USA), p16^{INK4a} (Klinipath, Duiven, the Netherlands), γH2AX (Millipore, Billerica, USA) and p53 (Dako, Glostrup, Denmark) the sections were deparaffinized in xylene and rehydrated with ethanol. Antigen retrieval was performed in a microwave for 15 minutes. Endogenous peroxidase activity was blocked and sections were washed with Tris/HCl pH8.0. The primary antibodies to Ki-67, p16^{INK4a}, ERα, γH2AX and p53 were applied at respectively 1:50, 1:100, 1:200, 1:500 and 1:400 dilutions and incubated at room temperature for 1 to 2 hours. After washing with Tris/HCl, sections were incubated for 30 min. at room temperature with

biotinylated secondary antibody (Dako, Glostrup, Denmark). Diaminobenzidine (DAB, Dako, Glostrup, Denmark) was used for visualization of antigen–antibody reactivity and was applied for 2 times 5 minutes. The slides were counterstained with hematoxylin for 30 seconds, and then dehydrated with alcohol and xylene.

Light microscopic evaluation was performed blinded. The difference in the mean number of positive cells in the epidermis per square millimeter for the different groups was calculated according to the Mann-Whitney test using SPSS version 15.0. A two-tailed *P*-value of <0.05 was chosen to represent statistical significance.

Quantitative real-time RT-PCR

Total RNA was isolated as described. Accordingly, cDNA was generated from 1 µg total RNA using T7 oligo d(T) primers (Invitrogen, Carlsbad, CA, USA) and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the Affymetrix protocol for first strand cDNA synthesis (Affymetrix, Santa Clara, CA, USA). Real-time quantitative RT-PCR (38 cycles, 15 sec. at 95°C, 30 sec. at 59-62°C and 1 min. at 72°C) was performed in duplicate using the Opticon I (Applied Biosystems, Foster City, CA, USA) and SYBR Green ITM (Applied Biosystems, Foster City, CA, USA). In short, cDNA samples (10ng each) were amplified with gene specific primer pairs (0.5 µM) in a total volume of 25 µl including 12.5 µl SYBR Green PCR master mix (Applied Biosystems, Applied Biosystems, Foster City, CA, USA). The housekeeping gene β-actin was used for normalization and all PCR primers used were intron spanning. The primer sequences used were as follows. *FANCA*: 5'- GCTTGAGGTAGAAGGTCCACT-3' (forward); 5'-CCTGCAAAGCAGAGCCTATAAAT-3' (reverse). *FANCD2*: 5'-GATGTC-CTTTCAAGCCTCCGA-3'(forward); 5'-CATCACCAACTGGCGAACC-3'(reverse). *BRCA1*: 5'-AGGCAACTTATTGCAGTGTGG-3' (forward); 5'-AATGAGCTGGCATGAG-TATTTGT-3' (reverse). *RAD51*: 5'-CTGAGGCAGCTAAATTAGTTCCA-3' (forward); 5'-CACCCCGGTCAATGGGAAG-3'(reverse). β-actin: 5'-TCCCTGGAGAAGAGCTACGA-3'(forward); 5'-AGGAAGGAAGGCTGGAAGAG-3' (reverse). The relative fold increase of real-time RT-PCR results was determined by the $2^{-\Delta\Delta Ct}$ method using the average expression of the housekeeping gene β-actin as a control.[19] Statistical analysis was performed with the use of the SPSS 15.0. After log transformation, a normal distribution was obtained and differences were calculated using the Mann-Whitney test. A two-tailed *P*-value of <0.05 was chosen to represent statistical significance.

RESULTS

Identification of differentially expressed genes in low-risk versus high- risk HPV infection

Unsupervised cluster analysis of control, condylomata and uVIN samples readily distinguished HPV positive disorders from normal control samples. Furthermore we observed a distinct pattern of gene expression between condylomata and uVIN samples (Figure 1).

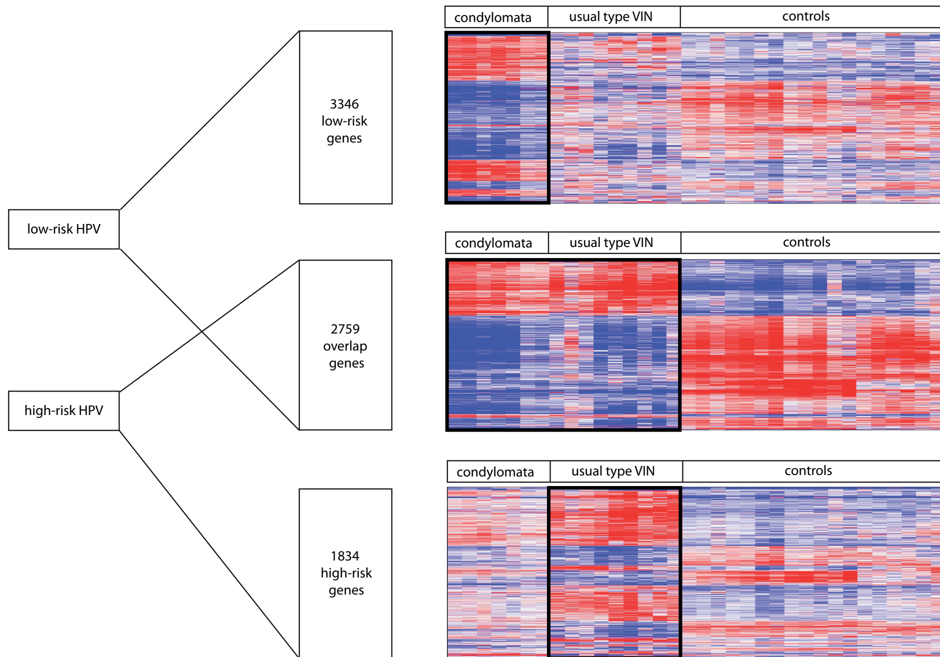


Figure 1. Gene expression profile. Unsupervised clustering of the expression profiles obtained from 18 control, 7 condylomata (low- risk HPV) and 9 usual typeVIN (high-risk HPV) samples. Firstly, ANOVA analysis "condylomata versus control" showed 6105 genes that were differentially regulated. From these genes, 3346 genes were exclusively regulated in condylomata, the so-called low-risk genes. Secondly, ANOVA analysis "uVIN versus control" showed 4593 genes that were differentially regulated. From these genes, 1834 genes were exclusively regulated in uVIN, the so called high-risk genes. In the middle of the figure, the 2759 overlap genes are visualized. These genes were differentially regulated in condylomata as well as in uVIN. Each row represents one gene (red: up regulated, blue: down regulated) and each column represents one expression profile obtained from one tissue sample.

To identify genes that were expressed in low- and/or high-risk HPV infections, we combined ANOVA analyses of "condylomata versus control" and "uVIN versus control". 6105 genes were identified that were expressed significantly different between condylomata and control samples and 4593 genes were identified between uVIN and control samples. In Figure 1 the combination of these two analyses is visualized. The overlap between the ANOVA analyses "condylomata versus control" and "uVIN versus control" was 2759 genes (Figure 1). These 2759 overlap genes (Figure 1B) are regulated by HPV, independent whether or not it is a low- or a high-risk HPV infection. Gene expression data used for Figure 1 can be accessed from supplementary Table 1 (http://www.erasmusmc.nl/47393/1584119/1603959/Santegoets).

An example of such an overlap gene is *ESR1* (Estrogen Receptor 1). We found a strong downregulation of *ESR1* in condylomata and uVIN in comparison to control

tissues (condylomata versus control: *P*<0.001 and uVIN versus control: *P*<0.001). This finding has also been reported for high-risk HPV induced cervical cancer[20] and was confirmed by immunohistochemistry for Estrogen Receptor α (ERα). ERα immunoreactivity was clearly present in normal tissue, while no or little staining was observed in condylomata and uVIN samples (condylomata versus control: *P*<0.001 and uVIN versus control: *P*<0.001) (Figure 2A). Because loss of ERα automatically results in loss of regulation of estrogen regulated genes in the vulva we have compared the 2759 overlap genes (Figure 1) with published data from literature on estrogen regulated genes in the endometrium.[21-22] It was observed that a number of reported estrogen target genes were indeed affected by the HPV induced downregulation of ERα (detailed information can be accessed from supplementary Table 2 (http://www.erasmusmc.nl/47393/1584119/1603959/Santegoets)). Some examples of such genes are *IGF1*, *IGFBP5*, *ADAMTS1*, *SFRP4*, *EDN3*, *FN1*, *COL1A2*, *BCAS1*, *IGFBP4*, *GALNT4*, *ACTA2* and *PTCH1*.

Additionally we could identify 3346 genes (Figure 1A) that were only differential expressed in low-risk HPV associated condylomata lesions (further called low-risk genes) and 1834 genes (Figure 1C) that were exclusively differential expressed in high-risk HPV associated uVIN lesions (further called high-risk genes). By studying these low- and high-risk genes, we were able to perform a functional analysis, as discussed below.

Functional analysis of differences between low- and high-risk HPV types

By using Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com) we compared the low-risk specific genes (studied in condylomata) with the high-risk specific genes (studied in uVIN) and we identified biological functions that were regulated significantly different between condylomata and uVIN. We selected the two most significantly different biological functions for further research: firstly it was observed that significantly more genes related to cell cycle were regulated in uVIN in comparison with condylomata; and secondly genes involved in the DNA damage response pathway were significantly higher regulated in uVIN than in condylomata. Gene expression data of genes involved in cell cycle regulation and genes involved in the DNA damage response can be accessed from Supplementary Table 3 (http://www.erasmusmc.nl/47393/1584119/1603959/Santegoets).

In Figure 3 we used the KEGG cell cycle pathway scheme as a background for our own microarray data on condylomata and uVIN. It is clear that many genes involved in regulation of cell cycle are highly modulated in uVIN and condylomata. The upregulation of different cyclins (Cyclin A (*CCNA*), B (*CCNB*) and E (*CCNE*)) in uVIN as well as condylomata, suggests that proliferation is enhanced in uVIN and condylomata. Therefore, proliferation was measured using immunostaining of the proliferation marker Ki67. The staining pattern of Ki-67 was nuclear and immunoreactivity in control tissue was located in the basal and parabasal layer, while in uVIN lesions Ki-67 staining was observed through-out the whole epidermis. A lower expression of Ki-67 was observed in normal tissue than in condylomata (*P*<0.001) and uVIN (*P*<0.001).

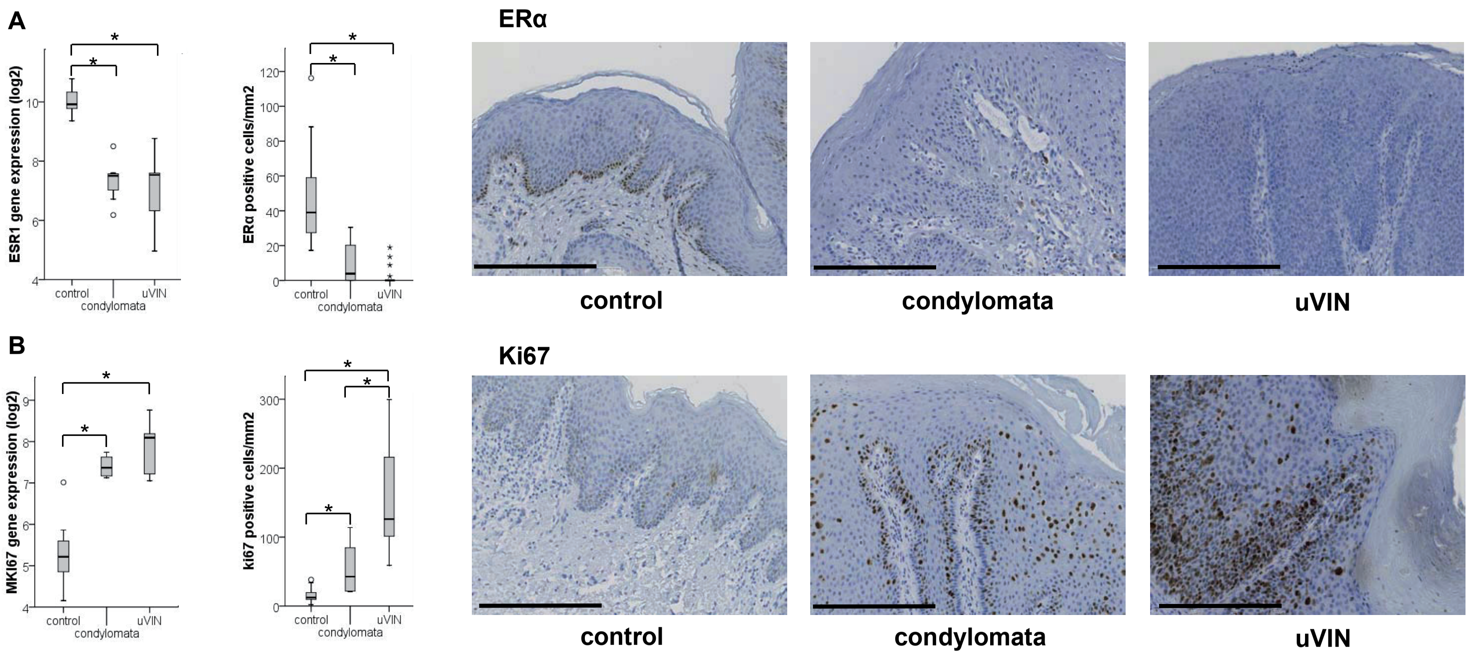
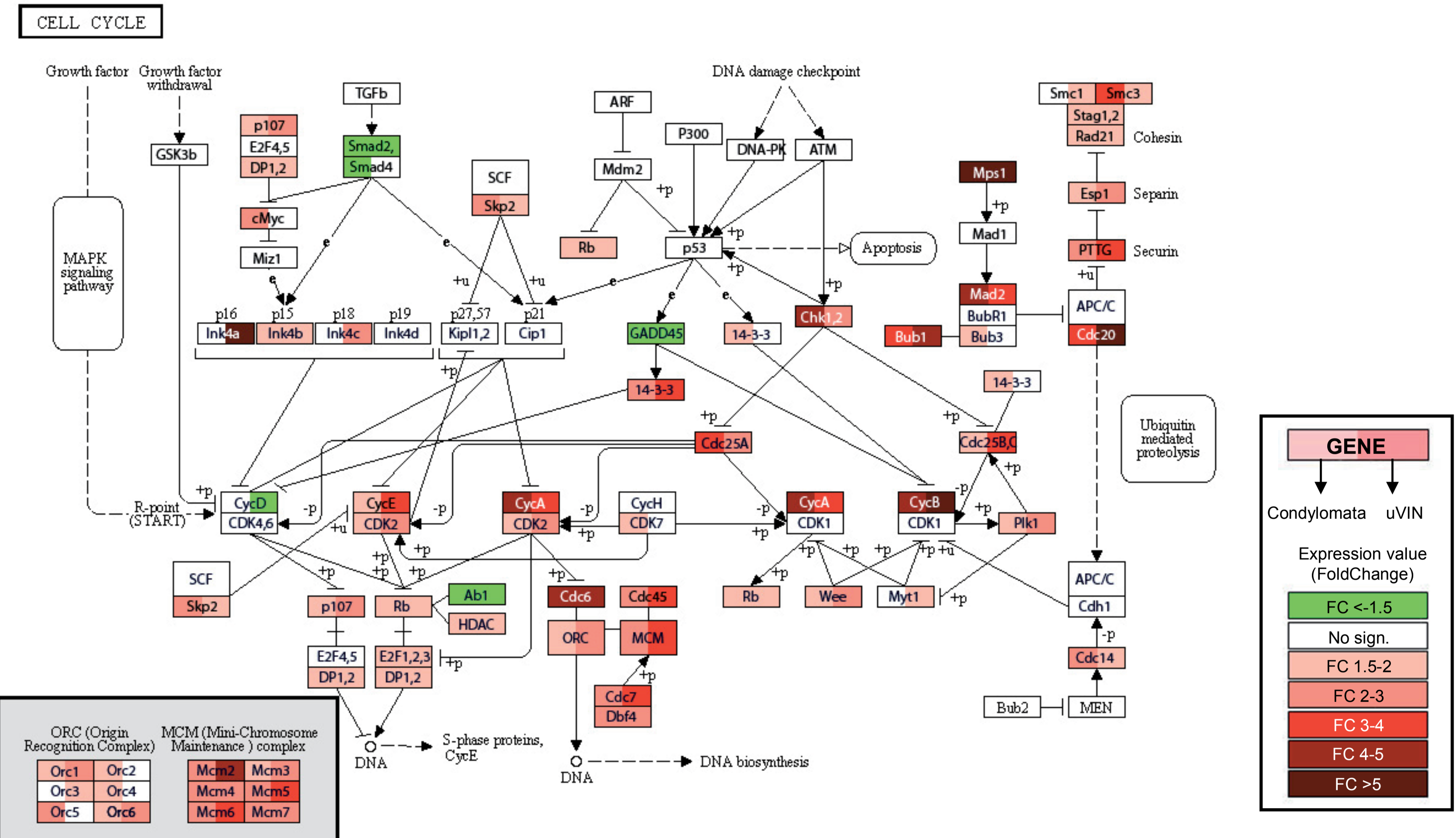


Figure 2. Correlation between mRNA and protein expression. Correlation between mRNA and protein expression of **A)** Estrogen Receptor α (*ESR1*), **B)** MKI67 (Ki67). On the left hand side of the figure, mRNA and protein levels are visualized: the first boxplot represents the mean level of gene expression, as measured on the microarrays, on a log2 scale. The second boxplot represents the mean number of positive cells per mm^2 epidermis. On the right hand side of the figure, representative images of ERα and Ki67 immunohistochemistry in control, condylomata and uVIN samples are shown. Magnification: 20X, scale bar represents 300μm. *: $P<0.05$.

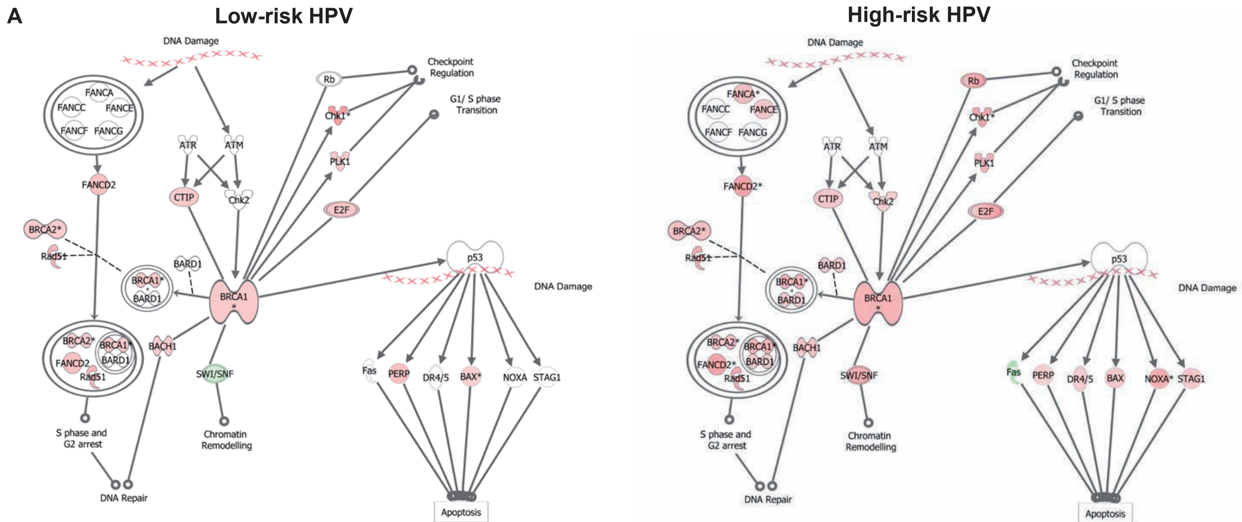

CELL CYCLE
Growth factor
Growth factor withdrawal
GSK3b
MAPK signaling pathway
R-point (START)
TGFb
Smad2,
Smad4
p107
E2F4,5
DP1,2
cMyc
Miz1
SCF
Skp2
ARF
Mdm2
Rb
P300
DNA damage checkpoint
DNA-PK
ATM
p53
Apoptosis
p16
Ink4a
p15
Ink4b
p18
Ink4c
p19
Ink4d
p27,57
Kipl1,2
p21
Cip1
GADD45
14-3-3
Chk1,2
Mps1
Mad1
Mad2
BubR1
Bub3
Bub1
Smc1
Smc3
Stag1,2
Rad21
Cohesin
Esp1
Separin
PTTG
Securin
APC/C
Cdc20
Cdc25A
Cdc25B,C
Ubiquitin mediated proteolysis
CycD
CDK4,6
CycE
CDK2
CycA
CycH
CDK7
CDK1
CycB
Plk1
Wee
Myt1
Cdh1
Cdc14
Bub2
MEN
Ab1
HDAC
E2F1,2,3
Cdc6
Cdc45
ORC
MCM
Cdc7
Dbf4
DNA
S-phase proteins, CycE
DNA biosynthesis
ORC (Origin Recognition Complex)
Orc1
Orc2
Orc3
Orc4
Orc5
Orc6
MCM (Mini-Chromosome Maintenance) complex
Mcm2
Mcm3
Mcm4
Mcm5
Mcm6
Mcm7
G1
S
G2
M
GENE
Condylomata
uVIN
Expression value
(FoldChange)
FC <-1.5
No sign.
FC 1.5-2
FC 2-3
FC 3-4
FC 4-5
FC >5

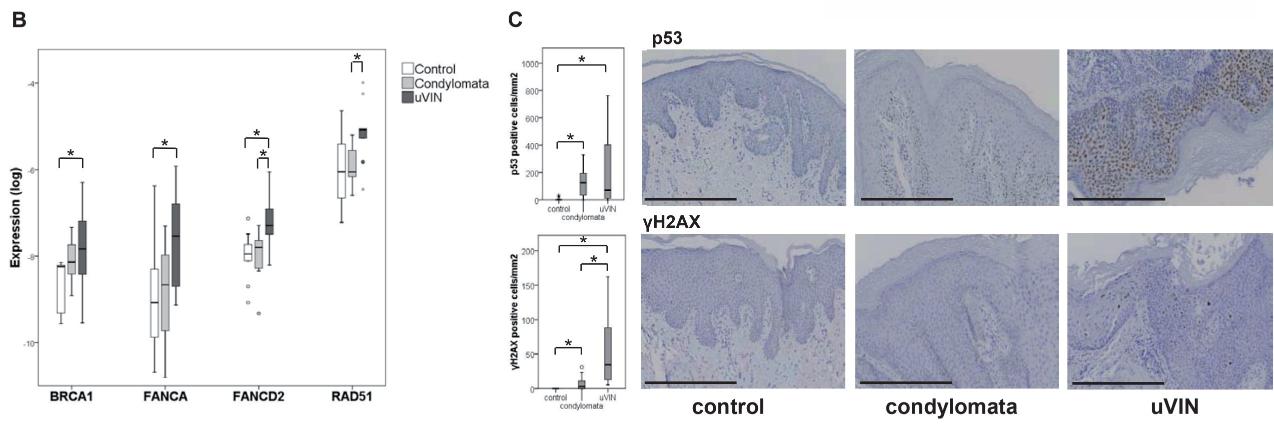
Furthermore, a lower level of Ki-67 positivity was also seen in condylomata compared to uVIN (*P*<0.001) (Figure 2B). This difference in proliferation might be explained by differences in gene expression of cell cycle genes between uVIN and condylomata.

Next to regulation of the cell cycle, the DNA damage response was observed to be significantly elevated in uVIN compared to condylomata. In Figure 4, DNA damage response genes differentially expressed between condylomata and uVIN (Figure 4A) are indicated. As reviewed in this figure, more genes are deregulated in high-risk HPV induced uVIN than in low-risk HPV induced condylomata. To verify regulation of a number of differentially regulated genes (*BRCA1*, *FANCA*, *FANCD2* and *RAD51*), quantitative RT-PCR was performed. In line with our microarray data, all these genes were significantly upregulated in uVIN compared to control (Figure 4B).

Based on the above, it was anticipated that a difference in DNA damage could be detected between low-risk HPV induced condylomata and high-risk HPV induced uVIN. To study this in more detail we perfomed γ-H2AX immunostaining. γ-H2AX is essential to the efficient recognition and repair of DNA double strand breaks and represents a sensitive marker for DNA damage in cells. It was observed that in control tissues no DNA damage could be detected, while both in condylomata as well as in uVIN, γ-H2AX positive cells were readily identified (Figure 4C). Furthermore, staining in high-risk HPV induced uVIN was significantly higher than in low-risk HPV induced condylomata. These results indicate a quantitative difference in DNA damage between high-risk and low-risk HPV induced disorders. To further substantiate this, p53 signalling was assessed by studying genes involved in the p53 pathway by using Ingenuity (for overview of the p53 pathway, see Supplementary Figure 1 (http://www.erasmusmc.nl/47393/1584119/1603959/Santegoets)). The tumor suppressor p53 detects DNA damage, resulting in cell cycle arrest or apoptosis. Although at RNA level p53 was not regulated, the expression of a number of genes was significantly changed in this p53 pathway (19 genes were upregulated and 8 genes downregulated in uVIN, while in condylomata only 10 genes were upregulated and 2 genes were downregulated (Table 1)). Immunostaining for p53 revealed no staining in control tissues, while in condylomata and uVIN p53 nuclear staining was observed. Likewise the amount of p53 positive cells was significantly higher in condylomata (*P*=0.001) and uVIN (*P*=0.002) than in the control group. In condylomata the nuclear staining pattern was observed mainly around the basal layer, while in uVIN p53 positive cells were observed throughout all layers of the epithelium (Figure 4C).

◁ **Figure 3.** Cell cycle regulation in condylomata and uVIN. The cell cycle scheme form the KEGG database (http://www.genome.jp/kegg/pathway/hsa/hsa04110.html) was used to overlay gene expression values for different genes in condylomata and uVIN. Each box represents one gene: the color on the left site of the box represents the expression value (Fold Change) in condylomata, and the right part of the box represents the expression value (Fold Change) in uVIN.


A
Low-risk HPV
High-risk HPV
DNA Damage
FANCA
FANCC
FANCE
FANCF
FANCG
FANCD2
BRCA2*
Rad51
ATR
ATM
CTIP
Chk2
BARD1
BRCA1* BARD1
BRCA1
BRCA2* BRCA1* FANCD2 BARD1 Rad51
BACH1
SWI/SNF
Chromatin Remodelling
S phase and G2 arrest
DNA Repair
Rb
Chk1*
PLK1
E2F
Checkpoint Regulation
G1/ S phase Transition
p53
Fas
PERP
DR4/5
BAX*
NOXA
STAG1
Apoptosis
FANCA*
FANCD2*
BAX
NOXA*


B
Expression (log)
BRCA1
FANCA
FANCD2
RAD51
Control
Condylomata
uVIN
C
p53
p53 positive cells/mm2
γH2AX
γH2AX positive cells/mm2
control
condylomata
uVIN

Identification of markers for high-risk HPV infected cells

In order to identify virus-specific, but tissue-unrelated markers of high-risk HPV infection, we compared our own data with two other studies.[23-24] A total of 120 gene expression profiles were available for comparison: 41 high-risk HPV positive cancers (24 squamous cell carcinomas of the head-and-neck and 17 cervical cancers), 62 HPV negative cancers (59 squamous cell carcinomas of the head-and-neck and 3 cervical cancers) and 17 healthy control tissues. Microarray data of these samples were obtained from the GEO DataSet option in PubMed under accession GSE6791 and GSE3292.

We performed the following ANOVA analyses:

1. HPV positive squamous cell carcinomas of the head-and-neck versus control tissue of head-and-neck: 1223 genes were significantly different expressed
2. HPV positive cervical cancer versus cervical control tissue: 338 genes were significantly different expressed
3. HPV positive squamous cell carcinomas of the head-and-neck versus HPV negative squamous cell carcinomas of the head-and-neck: 667 genes were significantly different expressed.

These data were compared to our own high-risk gene list (1834 genes, Figure 1, Supplementary Table 1 (http://www.erasmusmc.nl/47393/1584119/1603959/Santegoets)) and the overlap is visualized in Figure 5A.

Interestingly, by analyzing the microarray data of the study of Pyeon and Slebos [23-24] in comparison with our own data, only one gene was present in all three analyses, namely *CDKN2A*, which encodes the $p16^{INK4a}$ tumor suppressor protein. It is highly expressed in all high-risk HPV positive tissues, while it is not expressed in control tissues and only in a few HPV-negative classified disorders (Figure 5B).

We confirmed upregulation of $p16^{INK4a}$ with immunohistochemistry in our own samples. The staining pattern of $p16^{INK4a}$ was cytoplasmic and nuclear, with more nuclear than cytoplasmic staining. Normal tissue and condylomata showed either no or only minimal immunostaining for $p16^{INK4a}$, while all uVIN lesions were positive for $p16^{INK4a}$ (uVIN versus controls: $P<0.001$, Figure 5C).

◁ **Figure 4.** DNA damage pathway regulation in low- and high-risk HPV infection. Graphical representation of the DNA damage response pathway (using Ingenuity Pathway Analysis Software). Red indicates an up regulation of gene expression, whereas green indicates a down regulation. **A)** DNA damage response pathway regulation in a low-risk HPV infection (condylomata) and in a high-risk HPV infection (uVIN). **B)** Boxplots of mean number of positive cells per mm^2 epidermis for immunostaining for γH2AX and p53. Next to the boxplots, representative images of γH2AX and p53 immunohistochemistry in control, condylomata and uVIN are shown. Magnification: 20X, scale bar represents 300µm. **C)** Realtime RT-PCR results for *FANCA*, *BRCA1*, *FANCD2* and *RAD51*. Expression data were analyzed using the comparative CT method whereby the change in expression of the target gene relative to the housekeeping gene (β-actin) was calculated. On the y-axis expression values are represented on a log scale. Magnification: 20X, scale bar represents 400µm. *: $P<0.05$.

2.3

Table 1. Regulated genes involved in p53 pathway

Gene Title	Gene Symbol	FC uVIN vs controls	FC condylomata vs controls
upregulated			
cyclin-dependent kinase inhibitor 2A (p16)	CDKN2A	20.62	
proliferating cell nuclear antigen	PCNA	3.7	2.09
baculoviral IAP repeat-containing 5 (survivin)	BIRC5	3.19	3.25
CHK1 checkpoint homolog (S. pombe)	CHEK1	3.03	3.69
phorbol-12-myristate-13-acetate-induced protein 1	PMAIP1	2.76	1.71
breast cancer 1, early onset	BRCA1	2.66	1.93
protein kinase, DNA-activated, catalytic polypeptide	PRKDC	2.45	
cyclin-dependent kinase 2	CDK2	2.42	1.62
retinoblastoma 1 (including osteosarcoma)	RB1	2.11	
Stratifin	SFN	2.05	3.01
histone deacetylase 1	HDAC1	1.92	1.57
stromal antigen 1	STAG1	1.91	
chromosome 12 open reading frame 5	C12orf5	1.86	2.77
BCL2-associated X protein	BAX	1.8	
CHK2 checkpoint homolog (S. pombe)	CHEK2	1.67	
E2F transcription factor 1	E2F1	1.67	
tumor protein p63	TP63	1.63	1.97
hypothetical protein MGC5370	MGC5370	1.62	
PERP, TP53 apoptosis effector	PERP	1.5	
downregulated			
homeodomain interacting protein kinase 2	HIPK2	-1.54	
cyclin G1	CCNG1	-1.67	
phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	PTEN	-1.7	
reprimo, TP53 dependent G2 arrest mediator candidate	RPRM	-1.94	
cyclin D2	CCND2	-2.01	
pleiomorphic adenoma gene-like 1	PLAGL1	-2.56	-1.58
cyclin D1	CCND1	-4.06	
fatty acid synthase	FASN	-6.33	-4.65

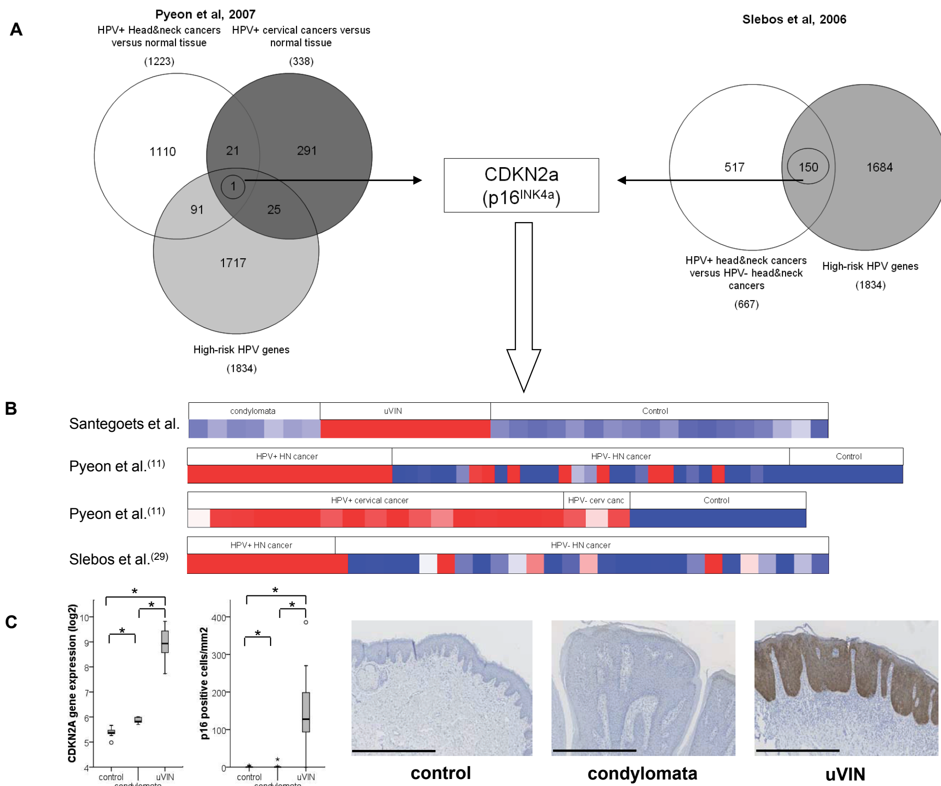


Figure 5. *CDKN2A* as a marker for high-risk HPV infection. **A)** Comparison of significantly regulated genes by high-risk HPV infection between different tissues. ANOVA reveals a list of genes significantly different between HPV positive cancers (Head&Neck and cervical) of the studies of Pyeon et al. [24] on the left and Slebos et al. [23] on the right. These gene lists are compared to our own high-risk gene list (1834 high-risk genes, visualized in Figure 1). Results of pairwise comparisons are summarized in the Venn diagrams. Only one gene, namely *CDKN2A*, is present in all high-risk HPV related disorders. **B)** *CDKN2A* is highly expressed in all high risk HPV positive disorders. Each row represents the expression value for *CDKN2A* in the different studies and each column represents one sample. The box above the row represents the group of disorders (HN: head and neck). Red: upregulation. Blue: downregulation. **C)** The left hand boxplot represents the mean level of gene expression of *CDKN2A*. The other boxplot represents the mean number of p16 positive cells per mm^2 epidermis. On the right hand of the figure, representative images of p16 immunohistochemistry of control, condylomata and uVIN are shown. Magnification: 10X, scale bar represents 800µm.*: $P<0.05$.

DISCUSSION

In the current investigations, significant differences in gene expression profiles between low-risk (studied in condylomata) and high-risk (studied in uVIN) HPV infections were observed. Upon analyzing biological processes affected by the observed gene expression differences, proliferation (cell cycle) and DNA damage response were more significantly regulated in high-risk HPV infections than in low-risk HPV infections.

As reviewed by Pim and Banks, the main difference in malignant potential between low- and high-risk HPV can be attributed to differences in the ability of oncoproteins E6 and E7 to induce transformation of cells.[6] Pim and Banks acknowledge that a limitation to their review is the fact that far more basic and clinical research is focused on high-risk HPV types than on low-risk types.

Here we will discuss the role of the oncoproteins E6 and E7 and the pathways in which they are involved in relation to our findings.

DNA damage response is activated by HPV

According to the literature, high-risk HPV-16 E7 oncoprotein induces deregulated S-phase entry causing replication stress, which ultimately results in DNA breakage.[25] For a relatively long period of time, HPV infected cells can limit the adverse effects of DNA damage by increased DNA damage repair mechanisms. In the current investigations we have observed a clear upregulation of DNA damage response in uVIN, and to a much lesser extent in condylomata, as shown in Figure 4. *BRCA1*, a tumor suppressor gene which is important in response to DNA damage, is in the center of this DNA damage control pathway and shows more pronounced upregulation in uVIN (supplementary Table 3 (http://www.erasmusmc.nl/47393/1584119/1603959/Santegoets) and Figure 4A). Similarly we found a strong upregulation of the Fanconi genes, *FANCA*, *FANCD2* en *FANCE* , both on micro-array and quantitative real-time RT-PCR. These genes are named after patients with Fanconi Anemia (FA), which is a recessively inherited disease that is characterized by congenital abnormalities, aplastic anemia and a predisposition for the development of cancer, particularly HPV related squamous cell carcinomas. The development of these carcinomas in patients with FA is described in a number of case-reports.[26-29] This increased risk for tumor development seems to be caused by FA deficiency in the DNA damage response pathway.[30-31] The role of HPV infection as an initiator in this process has been studied by Spardy et al..[32] They found that the DNA damage response pathway is activated by HPV-16 E7 oncoprotein, resulting in DNA damage repair and suppression of hyperplasia. These results were confirmed by Hoskins et al, who found that knockdown of *FANCA* or *FANCD2* in HPV infected keratinocytes resulted in increased epithelial hyperplasia, while complementation of these FA proteins caused reduced rates of proliferation.[33] From these data it was concluded that enhanced expression of high-risk HPV-16-E7 initially results in a higher activation of the DNA damage response pathway, in an effort to minimize DNA damage induced by a high-risk HPV infection induced E7 expression, which is in accordance with our findings.

Besides inducing repair, DNA damage will also result in activation of the p53 pathway. Activation of the p53 pathway is necessary for DNA-damage control because p53 activation can stall the cell cycle to allow for DNA repair, or induce apoptosis where DNA-damage is beyond repair.[34] Activation of the p53 pathway is related to the other oncoprotein mentioned above, namely E6.[35] We found that several genes acting in the p53 pathway showed more pronounced regulation in uVIN than in condylomata (Table 1). According to current literature immunohistochemical staining for p53, indicating stabilized wild-type or mutant p53, is frequently detected in vulvar cancer and differentiated VIN, but not in uVIN.[36-37] Nevertheless we found p53 diffusely expressed in uVIN lesions, while in condylomata positive cells were mainly observed around the basal membrane. These results suggest that due to an HPV infection, activation of DNA damage response results in activation of the p53 pathway. However, the fact that only a fraction of women infected with high-risk HPV will develop HPV-associated cancer, indicates that additional factors are needed for malignant progression.[38] Wei et al. found that nitric oxide (NO) can be such an additional factor. Increased NO levels have been associated with several known risk factors for cervical cancer, such as tobacco smoking, multiparity, long-term use of oral contraceptives, chronic inflammation and sexually transmitted disorders.[39] Wei et al. studied the effect of NO in HPV infected cells and found that NO exposure results in survival of HPV-infected cells harbouring serious DNA damage.[40] Thus in order for a persistent HPV infected tissue to proceed to malignancy, additional factors, for example smoking-induced NO, are needed to further inactivate p53.

Genes involved in cell cycle are deregulated by HPV, resulting in proliferation

As a result of increased DNA damage and resulting DNA repair, one would expect that progression through the cell cycle would become inhibited. However, this is not the case: increased proliferation was measured in uVIN as compared to condylomata and controls (Figure 2B and Figure 3). Previously we demonstrated this increase in proliferation and mentioned it as one of the hallmarks in the process of carcinogenesis.[18] A central role in inducing proliferation is played by oncoprotein E7. Overexpression of this protein can trigger activation of different cell cycle regulatory genes involved in the E2F transcription factor pathway, like *MCM*s, *ORC1*, *CDC7* and *PCNA*. Upregulation of these genes in HPV related disorders has been documented, suggesting that a persistent HPV infection affects the E2F transcription factor pathway.[23, 41-44] The central function of this pathway is regulating progression through the cell cycle. Recently, Spardy et al found that E7 inhibits DNA damage checkpoint control by inducing breakdown of claspin, a critical regulator of the *ATR/CHK1* signaling and DNA damage checkpoint recovery during cell division.[45]

In this study the main difference in cell cycle regulation between low-risk and high-risk HPV was the upregulation op *CDKN2A* (p16) in uVIN. This was observed in our microarray, but also by immunostaining. The importance of p16 will be discussed below.

p16^{INK4a} as a molecular marker for high-risk HPV infections

In order to rule out confounding effects related to tumor site and thus enabling us to study exclusively the effects of high-risk HPV infections, we assessed molecular changes in a variety of HPV positive lesions by comparing different microarray studies. A limitation of this approach was that we compared premalignant uVIN lesions with invasive cancers. However, by doing this, it was hypothesized that novel molecular markers for high-risk HPV infections might be detected. Surprisingly, only one gene was found to be exclusively expressed in all high-risk HPV disorders, namely *CDKN2A*, which encodes the p16^{INK4a} tumor suppressor protein. Increased levels of *CDKN2A* are highly correlated with the presence of high-risk HPV in cervical cancer, squamous cell carcinomas of the head-and-neck and uVIN and consequently this has been suggested as a surrogate marker for HPV.[46] Indeed, according to Figure 5, *CDKN2A* is a specific marker.

To conclude, we found significant differences between gene expression profiles of low- and high-risk HPV types. These differences are reflected in higher cell cycle progression and increased DNA repair mechanisms in high-risk HPV infections. Furthermore we confirmed that p16^{INK4a} is a highly specific molecular marker for high-risk HPV infections.

ACKNOWLEDGEMENTS

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3 NON-HPV RELATED EPITHELIAL DISORDERS OF THE VULVA

3.1

A RETROSPECTIVE STUDY OF 95 WOMEN WITH A CLINICAL DIAGNOSIS OF GENITAL LICHEN PLANUS

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J Low Genit Tract Dis, 2010;14(4):323-8

ABSTRACT

The aim of this retrospective clinical study was to evaluate clinical features, histopathology, treatment regimen and follow-up, in patients with genital lichen planus. Therefore, we retrospectively analysed data of 95 women diagnosed with genital lichen planus and visiting the vulvar clinic at Erasmus MC, Rotterdam, The Netherlands. All patients were symptomatic, most often complaining of vulvar soreness and burning (31.6%). Thirty-four percent of women had persistent symptoms for more than 5 years. On physical examination 81.1% showed sharply demarcated erythematous lesions, usually located at the vestibule and 56.8% had oral lesions. Treatment usually consisted of potent topical corticosteroids. Seventeen women (17.9%) were referred to the department of gynaecology for additional surgical treatment. In two of them a vulvar squamous cell carcinoma was detected, followed by radical surgery. In conclusion, in cases with vulvar soreness and burning, sharply demarcated erythematous vulvar lesions and the concomitant presence of oral lesions, the diagnosis of lichen planus should be considered and treatment must be initiated accordingly, even when histopathology is discordant.

INTRODUCTION

Lichen planus is a chronic inflammatory disease of skin and mucous membranes of unknown etiology, with some evidence of an autoimmune mechanism, whereby activated CD8+ T cells are directed against basal keratinocytes.[1-3] It is a relatively rare disorder. Its true prevalence is unknown, but it is estimated to effect 1% of the general population.[4] In 1982, the association of lichen planus of the vulva and vagina with desquamative gingivitis was recognized and termed *vulvo-vaginal-gingival syndrome*.[5]

This inflammatory dermatosis has a broad clinical spectrum. The pruritic, violaceous, polygonal papules are considered classic, although oral and genital mucous membrane lesions frequently occur as well.[1] Oral symptoms primarily consist of soreness and a burning sensation. Oral lesions are the only manifestation of lichen planus in 15-35 % of patients, but up to 40-53 % of patients with vulvo-vaginal lichen planus show oral involvement.[6-7] Di Fede et al. found that 87.1% of patients with oral lichen planus has concomitant vulvar lichen planus or/and lichen sclerosus lesions, suggesting that all women with clinical oral lichen planus should undergo a thorough multidisciplinary evaluation.[8] In comparison with the cutaneous form, in which the course is generally limited to less than 1 year, the mucosal form is often chronic.

Vulvar and/or vaginal lesions of lichen planus usually are a burden for patients. Although several treatment modalities including both topical and systemic agents are described, therapeutic measures are often ineffective.[9-10] Patients with genital lichen planus should be carefully monitored for possible malignant transformation.[11-12]

In addition to the studies of Cooper and Wojnarowska[13] and Kennedy and Galask,[14] we describe clinical features (e.g. concomitant presence of oral lesions), histopathology, treatment regimen and follow-up in 95 patients with genital lichen planus.

MATERIALS AND METHODS

From May 1995 to December 2002 we included 95 women with genital lichen planus, presenting at the vulvar clinic of the Department of Dermatology and Venereology, or the outpatient clinic of the Department of Obstetrics and Gynaecology, Erasmus MC, University Medical Center Rotterdam, the Netherlands. The vulvar clinic is operated by a dermatologist who was formerly trained as a gynaecologist (W.I.v.d.M.).

All patients were seen by the same physician (W.I.v.d.M.). A thorough inspection of genital skin and mucosa, oral mucosa and, if indicated, non-genital skin was performed. Material for histopathological examination of the genital lesions, if not performed earlier, was obtained by a 4 mm punch biopsy. Medical history, physical examination, histological findings, and treatment outcome in all women with a clinical diagnosis of genital lichen planus was reviewed. This clinical diagnosis was

established in the patient when bilateral, sharply demarcated erythematous lesions at the vestibule (Figure1A), and/or Wickham's striae at the vestibule or labia minora, and/or sharply demarcated erosions at the vaginal wall were observed.

Characteristic histopathological findings include compact orthokeratosis, wedge-shaped hypergranulosis, irregular acanthosis, vacuolar alteration of the basal layer, and a band-like lymphocytic infiltrate in close approximation to the dermis. Necrotic keratinocytes, also referred to as colloid, hyaline, cytoid, or Civatte bodies, are frequently seen in the lower epidermis and especially in the papillary dermis.[15] Patients with a classic gross appearance of lichen planus were included despite a histopathological diagnosis not verifying the lichen planus, since the results of histopathological examinations are sometimes misleading. In total, the medical records of 95 patients were reviewed. The following data were collected: age, original diagnosis, nature and duration of symptoms, previous and recent treatments, time of most recent sexual intercourse, the occurrence of autoimmune disease, and contact allergies and duration of follow-up. Physical examination data included the presence of anogenital lesions, presence of oral and/or skin lesions and posttreatment physical findings.

Data were analysed using descriptive statistics, presented in frequencies in text and tables. Approval for this retrospective study was given by the institutional review board.

RESULTS

Ninety-five women, aged 24 to 80 years (median: 55 years), with the clinical diagnosis of genital lichen planus, were evaluated in this study. Sixty-one women (64.2%) were postmenopausal. Of all 95 women, 38 (40.0%) were referred to our clinic by gynaecologists, 24 women (25.3%) by general practitioners, 20 women (21.1%) by dermatologists and 7 women (7.4%) by other healthcare providers (e.g. dentists, sexologists). Six women (6.3%) came on their own initiative.

In 50 women (52.6%) the diagnosis was 'uncertain' when they visited our clinic. Twenty-five women (26.3%) were referred with an already established diagnosis of

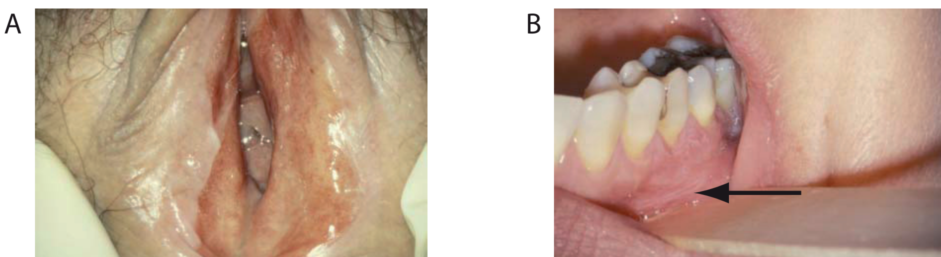

Figure 1. Clinical pictures of lichen planus. A: Sharply, demarcated glazed erythema at vaginal vestibule. B: White, reticulated mucosal lesions near molars.

lichen planus. In the remaining women 'other' diagnoses were established, such as lichen sclerosus (n=11).

All patients were initially symptomatic and complained often of vulvar soreness and burning (31.6%). On direct questioning, additional complaints such as dyspareunia, vulvar pruritus, contact bleeding, bleeding gums, inability to engage in intercourse, vaginal discharge and dysuria, also emerged (Table 1).

In 24 women (25.3%) symptoms had been present for a period of between 2 and 5 years (Table 2). Forty-two women (44.2%) had abstained from vaginal intercourse for at least a year.

The medical history of our patients further revealed that 16 women (16.8%) had contact allergies, with nine of them being allergic to nickel. Symptoms of atopic disease (hay-fever, asthma/bronchitis or eczema) were reported by 14 women (14.7%). Autoimmune disease was reported by 4 women (3 women had thyroid disease and 1 woman had vitiligo). Ten women (10.5%) had symptoms of cutaneous lichen planus in their medical history.

On genital examination, 77 patients (81.1 %) showed sharply demarcated erythematous lesions, usually located at the vestibule (69.5%). Fourteen women

Table 1. Complaints at first visit in women with genital lichen planus (n=95).

Complaint	number of women	%
Dyspareunia	51	53.7
Vulval soreness and burning	47	49.5
Vulval pruritus	46	48.4
Contact bleeding	16	16.8
Easily bleeding gums	13	13.7
Inability to engage in intercourse	11	11.6
Vaginal discharge	8	8.4
Dysuria	7	7.4

Table 2. Duration of complaints in women with genital lichen planus (n=95).

Duration of complaints	number of women	%
0-1 year	16	16.8
1-2 years	18	18.9
2-5 years	24	25.3
5-10 years	19	20.0
>10 years	13	13.7
unknown	5	5.3

(14.7%) showed one or more hyperkeratotic areas. Wickham's striae were present in 34 patients (35.8%), mostly (73.5%) located at the labia minora. In 16 women (16.8%) it was impossible to introduce a speculum, because of a partial or complete obliteration of the vagina, due to adhesions.

Oral inspection showed lesions (Wickham's striae, erosions and/or erythema) in 54 patients (56.8%). Wickham's striae were usually found at the gums near molars (Figure1B).

The location of the mucosal and skin lesions is presented in Table 3.

Ten women (10.5%) had lesions on the skin consistent with lichen ruber planus. Eight of these women had lesions on the trunk or extremities and two had lesions on the scalp (lichen planopilaris).

Histopathological examination was undertaken in 81 patients (85.3%). Biopsies were either taken from just outside erythematous lesions (usually located at the vestibule) or from lesions with Wickham's striae. In 12 patients (12.6%) histopathological examination had already been conducted at another hospital. A histological picture consistent with lichen planus was observed in biopsies from 9 of these women. In 2 women a biopsy was unfortunately not performed. However, in these women there was a strong clinical suspicion of lichen planus (erythematous lesions, Wickham's striae and characteristic lesions at the oral mucosa). In 72 patients (75.8%) the pathologist's report was consistent with the clinical diagnosis of lichen planus. Other diagnoses were: chronic non-specific inflammation, lichen sclerosus and 'uncertain' (Figure 2).

In our study 27 women (28.4%) were initially treated with a moderately potent topical corticosteroid (fluticasone propionate 0.005% ointment) and 64 women (67.4%) were treated with a highly potent topical corticosteroid (clobetasol propionate 0.05% ointment), usually twice daily for a month, whereafter tapering to a maintenance treatment of 2 to 3 times per week. Application site depended on the findings of the genital examination and was both vaginal and/ or vulvar. Four women (4.2%) were treated with intra-vaginal 10.0% hydrocortisone acetate in oestrogen cream. Sixty-seven women (70.5%) showed a slight to moderate improvement. Twenty-eight women (29.5%) showed no improvement at all. None of our patients was treated with systemic medication such as corticosteroids, cyclosporin or retinoids.

Table 3. Mucosal sites involved in women with genital lichen planus (n=95).

Location of lesions	number of women	%
Vulva, vagina and oral cavity	27	28.4
Vulva and oral cavity	24	25.3
Vagina and oral cavity	3	3.2
Vulva and vagina	3	3.2
Only vulva	34	35.8
Only vagina	4	4.2

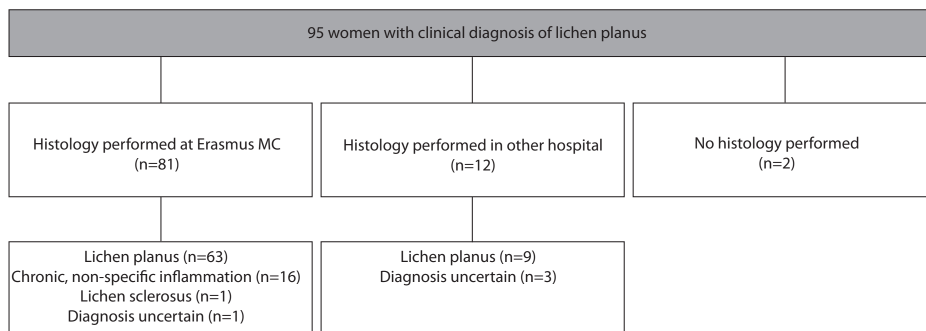

Figure 2. Histopathology. Results of histopathological examination in 95 women with a clinical diagnosis of genital lichen planus.

Seventeen women (17.9%) were referred to the gynaecological department of our hospital for additional surgery. Nine women, with a complete or partial vaginal obliteration, underwent an adhesiolysis of the vagina. During the post-operative period the frequent use of a vaginal dilator covered with corticosteroid cream was advised. In six patients minor introital plastic surgery was performed to alleviate symptoms of superficial dyspareunia. During follow-up a malignancy was suspected in two women, who both had a histologically proven diagnosis of lichen planus. Histopathological examination was repeated and showed an invasive carcinoma. These women were referred to the gynaecological department and underwent radical surgery.

Approximately half of the women with genital lichen planus are still followed-up at the vulvar clinic of the Erasmus MC. Most of the others were referred back to their own health care provider.

DISCUSSION

Lichen planus is a relatively rare disease. It must be considered in middle-aged women with symptoms of chronic dyspareunia, vulvar soreness, burning and oral lesions.

The disease commonly develops in the sixth decade, with a mean age of onset between 49 and 57 years.[15-17] This is corroborated by our study, in which the mean age was 55 years.

The coexistence of genital lichen planus with lesions at the oral mucosa was reported in several studies and has been described as the vulvo-vaginal-gingival syndrome.[16, 18-19] In our study 13 women complained of easily bleeding gums on direct questioning. Oral lesions, usually present as white, reticulated, or net-like areas at the gingival mucosa (Wickham's striae), were reported in 54 women (56.8%). This clearly shows that it is extremely important to pay particular attention to the examination of the oral cavity in patients with suspected genital lichen planus. The concomitant presence of oral and genital lesions is helpful in the diagnosis. The histopathological

confirmation of a clinical diagnosis of lichen planus with a vulvar or vaginal biopsy is sometimes cumbersome. In the presence of frank erosions the histopathological results of a biopsy may be misleading because of the absence of epithelium and thus variable histological features.[1] The biopsy should preferably be taken adjacent to the edge of an erythematous lesion. In case of presence of Wickham's striae, these should certainly be biopsied. Wickham's striae are believed to be caused by a focal increase in the thickness of the granular layer of the epidermis.[20] In our study 75.8% of the clinical diagnoses were confirmed by a pathologist. Lichen sclerosus, especially the early stages, may show considerable histopathological overlap with lichen planus.[21] The results of histopathological examination can be discordant, even when the clinical features are typical for lichen planus. However, in case of a strong clinical suspicion, this should prevail over the results of histolopathological examination and treatment must be initiated accordingly. On the other hand, it is possible that on rare occasions, one has to reconsider the clinical diagnosis at some point during the follow-up period.

With regard to the treatment of lichen planus, multiple therapeutic options, such as local and systemic corticosteroids[6,13], PUVA cream photochemotherapy[22], retinoids[23], oral cyclosporine[24], oral methotrexate[25] and -more recently- tacrolimus ointment[26-27] have been reported. Nevertheless it seems that treatment is often difficult and unsatisfactory, but application of a highly potent topical corticosteroid should be utilized initially. Additionally, topical immunosuppressive agents (i.e. tacrolimus (Protopic®) and pimecrolimus (Elidel®)) may be considered if topical steroids are not helpful.[26-29] However, to investigate the efficacy of these and other therapeutic modalities, randomised and placebo-controlled trials are necessary. One of the difficulties in undertaking such trials is the relative rarity of the disease and limited number of patients. To perform such a study the participation of multiple centers would be necessary.

Narrowing and/or shortening of the vagina and painful introital mucosal lesions often prevent intercourse in this population. In severe cases, and if the patient wants to resume sexual activity, it may be necessary to perform an adhesiolysis of the vagina. This recommendation is also suggested by others.[30-32] During the post-operative period the frequent use of vaginal dilators covered with a highly potent corticosteroid cream or tacrolimus ointment is indicated. In cases of long-lasting sexual problems, referring the patient to a sexologist may be worthwhile.

In this study there were two cases of squamous cell carcinoma, indicating that strict follow-up on a long-term basis is necessary, because of possible malignant transformation. Biopsy of any atypical lesion is essential to exclude malignancy.

In conclusion, an accurate diagnosis and follow-up of vulvo-vaginal lichen planus is important. In patients with a suspicion of vulvo-vaginal lichen planus, it is important to examine the oral cavity, especially the gingival mucosa, for the presence of disease. Multiple treatment options are available, but the results, thus far, are discouraging.

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3.2

AN AUTOIMMUNE PHENOTYPE IN VULVAR LICHEN SCLEROSUS AND LICHEN PLANUS: A TH1 RESPONSE AND HIGH LEVELS OF MICRORNA-155

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Submitted for publication

ABSTRACT

Vulvar lichen sclerosus and lichen planus are T-cell mediated chronic skin disorders. Although autoimmunity has been suggested, the exact pathogenesis of these disorders is still unknown.

Therefore, the aim of the current study was to investigate the molecular and immunological mechanisms critical in the pathogenesis of vulvar lichen sclerosus and lichen planus. By using gene expression profiling and real-time RT-PCR experiments, we demonstrated a significantly increased expression of the pro-inflammatory cytokines (*IFNγ*, *CXCR*3, *CXCL9*, *CXCL10*, *CXCL11, CCR5, CCL4, CCL5*) specific for a Th1 IFNγ induced immune response. In addition, *BIC*/microRNA-155 (miR-155) – a microRNA involved in regulation of the immune response – was significantly upregulated in lichen sclerosus and lichen planus (9.5- and 17.7- fold change respectively). Immunohistochemistry showed a significant T cell response, with pronounced dermal infiltrates of $CD4^+$, $CD8^+$ and $FOXP3^+$ cells. Furthermore, we observed high levels of autoantibodies in 34/106 lichen planus and in 6/42 lichen sclerosus patients. In conclusion, these data demonstrate an autoimmune phenotype in vulvar lichen sclerosus and lichen planus, characterized by increased levels of Th1 specific cytokines, a dense T cell infiltrate, enhanced *BIC*/miR-155 expression and the presence of autoantibodies.

INTRODUCTION

Vulvar lichen sclerosus (LS) and lichen planus (LP) are both chronic inflammatory skin disorders that have a negative impact on quality of life and may proceed to malignant disease.[1]

LS can affect any part of the skin, but is most frequently seen in the anogenital area. Although it can occur at all ages, LS is most common in the fifth to sixth decade.[2-3] Women with vulvar LS often present with severe pruritus and soreness of the vulvar and perianal area.[2-4] Typically, lesions appear as a figure-of-eight pattern around the vulva and anus, with white atrophic lesions or hyperkeratosis. In advanced stages, there is destruction of the vulvar anatomy, characterized by resorption of the labia minora, narrowing of the introitus and a buried clitoris.[3]

LP also generally develops in the fifth to sixth decade.[5-6] In contrast to LS, the mucosa can be involved in LP, resulting in vaginal and oral lesions.[7-8] Patients usually present with vulvar soreness, pruritus, burning, dyspareunia, and/or vaginal discharge. Erosive lesions, with denuded epithelium associated with typical white Wickham's striae, can arise at the vulvar or vaginal mucosa, and alterations of normal vulvar architecture may occur. In severe cases, adhesions and stenosis can lead to complete obliteration of the vagina.[5-7]

Histologically, both disorders are characterized by a band-like lymphocytic infiltrate and a thinned epidermis with vacuolar changes in the basal layer. In classic LS the lymphocytic infiltrate is located under a band of homogenized collagen below the dermoepidermal junction, while in LP this infiltrate is situated directly beneath the epidermis.[1]

Local application of ultrapotent corticosteroids is still the treatment of choice for both disorders, but this only reduces symptoms and generally does not stop progression.[5-6, 9-10] Consequently, life-long follow-up of these patients is indicated, since progression to vulvar squamous cell carcinoma has been reported for LS as well as LP.[1]

The etiology of these disorders has not yet been elucidated. Various studies have suggested different etiological factors. Cases of familial LS and LP have been reported and an association with HLA class II antigens has been found, suggesting a genetic background.[11-13] Other studies have suggested a role for local factors and infectious agents, such as an infection with Borrelia burgdorferi, human papillomavirus (HPV) or hepatitis C virus.[14-16] However, the results of these studies are inconclusive. Furthermore, patients with LS and LP are more frequently diagnosed with autoimmune disorders such as vitiligo and thyroid disease, and the presence of autoantibodies has been observed. This suggests the possibility of an autoimmunological basis for both disorders.[17]

In summary, since it is not clear what causes LS or LP, the aim of this study was to elucidate the molecular mechanisms involved in the pathogenesis of vulvar LS and LP.

METHODS

Patient samples

Women with histologically and clinically confirmed LS and LP were included. Two punch biopsies of the affected vulvar skin were collected. One was formalin-fixed and examined for histological diagnosis by an experienced pathologist (P.C.E.). The other was directly frozen in liquid nitrogen and stored at -80°C until further analysis. None of the women had been using topical corticosteroids in the 6 weeks before time of biopsy. Control samples were obtained from healthy women who underwent elective vulvar surgery for cosmetic reasons.

The medical ethical committee of the Erasmus University Medical Center approved our study design and all women voluntarily gave written informed consent.

Microarray and microarray data analysis

Affymetrix U133plus2 GeneChips were used to obtain gene expression profiles of eight LS, five LP and 14 control samples. Isolation of RNA, staining, washing and scanning procedures were performed as described by the manufacturer (Affymetrix, Santa Clara, CA, USA).[18] Raw (.CEL files) and normalized microarray data have been deposited in the GEO repository at NCBI under accession number GSE5563.

Differentially expressed genes were identified by using statistical analysis of microarrays (SAM). A False Discovery Rate (FDR) less than 1% was considered statistically significant. Using OmniViz software hierarchical clustering of differentially expressed genes was performed.

Ontological analysis, using Ingenuity Pathway software (Ingenuity® Systems, www.ingenuity.com), was employed to assess the functional relevance of the observed differences in gene expression profiles.

Quantitative real-time RT-PCR

IFNγ, IL17A, IL17F, IL22RA1 and *CXCL10*. Validation of the expression of these genes was performed in eight LS, eight LP and eight control samples in duplicate using real-time quantitative RT-PCR (38 cycles, 15 sec at 95°C, 30 sec at 59-62°C and 1 min at 72°C) using the Opticon I (Applied Biosystems, Foster City, CA, USA) and SYBR Green ITM (Applied Biosystems). cDNA samples (10 ng each) were amplified with gene specific primer pairs (0.5 µM) in a total volume of 25 µl including 12.5 µl SYBR Green PCR master mix (Applied Biosystems). The housekeeping gene β-actin was used for normalization and all used PCR primers were intron spanning. The used primer sequences were as follows.

β-actin: 5’-TCCCTGGAGAAGAGCTACGA-3’(forward);
5’-AGGAAGGAAGGCTGGAAGAG-3’ (reverse).
IFNγ: 5’-TGACCAGAGCATCCAAAAGA-3’(forward);
5’-CATGTATTGCTTTGCGTTGG-3’ (reverse).
IL17A: 5’-ACCAATCCCAAAAGGTCCTC-3’(forward);
5’-GGGGACAGAGTTCATGTGGT-3’(reverse).

IL17F: 5'CCTCCCCCTGGAATTACACT-3'(forward);
5'-TTCCTTGAGCATTGATGCAG-3'(reverse).
IL22RA1: 5'ACCCCAGACACGGTCTACAG-3'(forward);
5'-GTAGAGCTCCGTGAGGTTGC-3'(reverse).
CXCL10: 5'-CCACGTGTTGAGATCATTGC-3'(forward);
5'-TTCTTGATGGCCTTCGATTC-3'(reverse).

The relative fold increase of real-time RT-PCR results was determined by the $2^{-\Delta\Delta Ct}$ method[19] using the average expression of the housekeeping gene (β-actin) as a control.

MicroRNA-155 (miR-155). Validation of the expression of miR-155 was performed using a two-step TaqMan RT-PCR assay (Applied Biosystems) according to manufacturers' protocols in five LS, five LP and five control samples. The TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) was used for the preparation of cDNA; 10 ng of total RNA was used per 15 µl RT reaction. Reverse transcription was performed in a PTC-200 Peltier Thermal Cycler (MJ Research, Biozym, Landgraaf, Belgium) for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C. The RT products were subsequently amplified with sequence-specific human miRNA primers using the Applied Biosystems 7900HT Real-Time PCR system. The reactions were incubated in duplicate in a 96-well plate at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min. Amplification signals were computed with the SDS v.2.3 software (Applied Biosystems). The relative fold increase of miR-155 to control was determined by the $2^{-\Delta\Delta Ct}$ method[19] using the average expression of two endogenous controls, RNU44 and RNU48.

Immunohistochemistry

Immunohistochemical staining was performed on 6 µm thick frozen tissue sections. The following markers were used: CD4, marker for T helper cells (MT.310; Dako, Glostrup, Denmark); CD8, marker for cytotoxic T cells (DK25; Dako); FOXP3, marker for regulatory T cells (Treg) (EBioscience Ltd., Hatfield, UK) and CD19, marker for B cells (Beckmann Coulter, Brea, USA), in dilutions of respectively 1:50, 1:50, 1:50 and 1:25. Staining was performed as described previously.[20]

For identification of immature dendritic cells (DCs)/ Langerhans cells, staining for CD1a (clone O10, Immunotech, Marseille, France) in a dilution of 1:10 was performed on paraffin-embedded tissue sections as described earlier.[21]

Stained cells were counted throughout the entire epidermal thickness and 100 µm deep into the dermis in a blinded session. Because the cell infiltrate in LS tissue is localized in the mid-dermis below a subepidermal sclerotic zone, LS tissues were counted 100 µm deep into the mid-dermal infiltrate. After measuring the total area of the epidermis and dermis by using the Leica Image Analysis System, the number of cells per square millimeter was calculated for each layer separately.

Data collection on autoantibodies

We retrospectively reviewed patients with a clinical diagnosis of LS or LP who visited the vulvar clinic of the Erasmus MC between 1990 and 2010 and in whom autoantibody testing was performed. The following autoantibodies were studied: antinuclear antibodies (ANA), antithyroid peroxidase antibodies (anti-TPO) and antithyroglobulin antibodies (anti-Tg).

Statistical analysis

Data analysis was performed with the use of the SPSS 17.0 software package for Windows. Immunohistochemistry and PCR data were analyzed using the non-parametric Kruskal-Wallis test and the Mann-Whitney test for independent samples. Correlation studies were performed with the non-parametric Spearman's correlation coefficient. A two-tailed P value of $P < 0.05$ was chosen to represent statistical significance.

RESULTS

Identification of differentially expressed genes in LS and LP

To study signaling pathways involved in both disorders, we performed gene expression arrays. Using SAM analysis we found 6643 differentially expressed probesets between LS and controls, 7354 between LP and controls and 623 between LS and LP (Figure 1). The number of differentially expressed genes between LS and LP was only 10% of the number of genes differentially expressed between LP and controls, or between LS and controls (the complete list of differentially expressed genes can be obtained from supplementary Table 1 (http://www.erasmusmc.nl/47393/1584119/1603959/Santegoets)).

By using Ingenuity Pathway Analysis we identified biological functions that were significantly regulated in LS and LP as compared to control samples. The following biological functions were found ($P < 0.001$): 1) Antigen presentation, 2) Cell-mediated immune response, 3) Humoral immune response and 4) Inflammatory response (supplementary Figure 1 (http://www.erasmusmc.nl/47393/1584119/1603959/Sante-goets)). High regulation of these biological functions is caused by the fact that most differentially expressed genes between LS/LP and control tissues are involved in pathways related to the immune response.

In order to investigate the immune response in more detail, we used the KEGG cytokine-cytokine receptor pathway (www.genome.jp/kegg/) as a background for our own immune signaling data. Many cytokines were upregulated in LS and LP (Figure 2). For most genes this upregulation was more pronounced in LP than in LS. By studying those cytokines in more detail, we focused on well-known pro-inflammatory and anti-inflammatory cytokines.[22] Of the pro-inflammatory cytokines we found upregulation of *IL1*, *IL6* (only in LP), *IL7*, *IL15*, *IFNγ* and *TNFα*. For most of the anti-inflammatory cytokines no modulation was found (for example for *IL4* and *IL13*), and

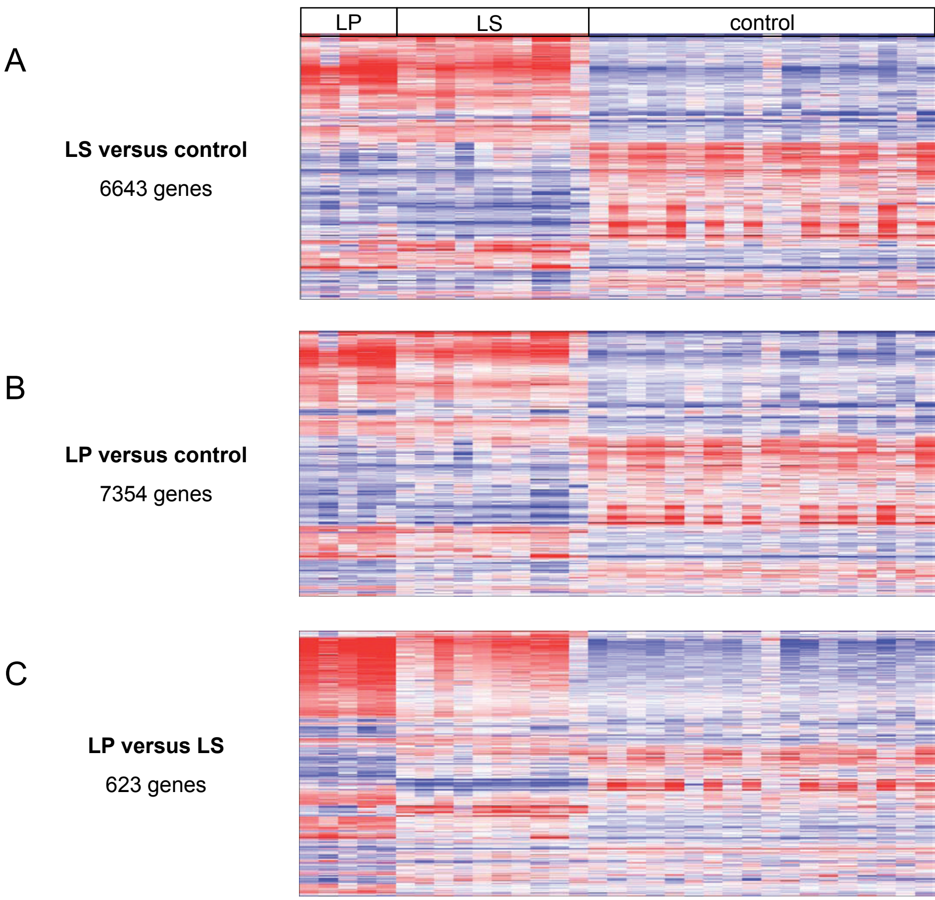

Figure 1. Gene expression profiles of lichen sclerosus (LS) and lichen planus (LP). Unsupervised clustering of expression profiles from 18 control, 5 LP and 10 LS samples. **A.** 6643 genes were differentially regulated in LS versus control. **B.** 7354 genes were differentially regulated in LP versus control. **C.** 623 genes were differentially regulated in LP versus LS. Each row represents one gene (red: upregulated, blue: downregulated), each column one expression profile from one tissue sample.

only slight upregulation of *TGFβ1* (only in LP) and *TGFβ2* (only in LS). Interestingly *IL11*, also known as an anti-inflammatory cytokine, was downregulated in LS and LP. In summary, significantly more pro-inflammatory cytokines were highly expressed in LS and LP, which is regarded as an indication for a cellular T helper type 1 (Th1) response.[23]

IFNγ is one of the key players in the Th1 response and its mRNA was strongly upregulated in LS and LP (3.5- and 7.8-fold, respectively, Figure 2). It is produced by T helper cells ($CD4^+$ T cells), which are characterized by chemokine-receptors *CXCR3* and *CCR5*.[24] As shown later (Table 1), in the dermis significantly higher levels of $CD4^+$

Figure 2. Cytokine - cytokine receptor pathway in lichen sclerosus (LS) and lichen planus (LP). The Cytokine-cytokine receptor scheme form the KEGG database (www.genome.jp/kegg/) was used to overlay gene expression values in LS and LP compared to controls. Each box represents one gene: the color represents the fold change expression value in LP (left side) and LS (right side). Black boxes mark IFNγ, CXCR3 and CCR5 and their ligands.

T cells were seen in LS and LP, and as expected both *CXCR3* and *CCR5* were highly expressed in both disorders. Besides this strong expression of *CXCR3* and *CCR5*, their activating ligands *CXCL9*, *CXCL10*, *CXCL11* and *CCL3, CCL4,* respectively, were also strongly upregulated. These results indicate that the infiltrate of $CD4^+$ T cells seems to be involved in the production of significant amounts of IFNγ, *CXCL9*, *CXCL10*, *CXCL11, CCL3* and *CCL4,* thus further stimulating a cellular Th1 response in LS and LP.

All the observations described above suggest a strong Th1 response. However, to investigate the potential role of Th2 and Th17 next to Th1, we performed real-time RT-PCR on important regulators of these responses. A clear and pronounced upregulation of *IFN*γ and *CXCL10* was confirmed, indicating a Th1 response, while we found no significant regulation of key players in the Th2 (*CCR3*, *IL4*) and Th17 (*IL17A*, *IL17F*, *IL22R*) response (Figure 3A).

One other important finding concerning the immune response was the strong upregulation of *BIC*, a gene which encodes for miR-155. MiR-155 plays

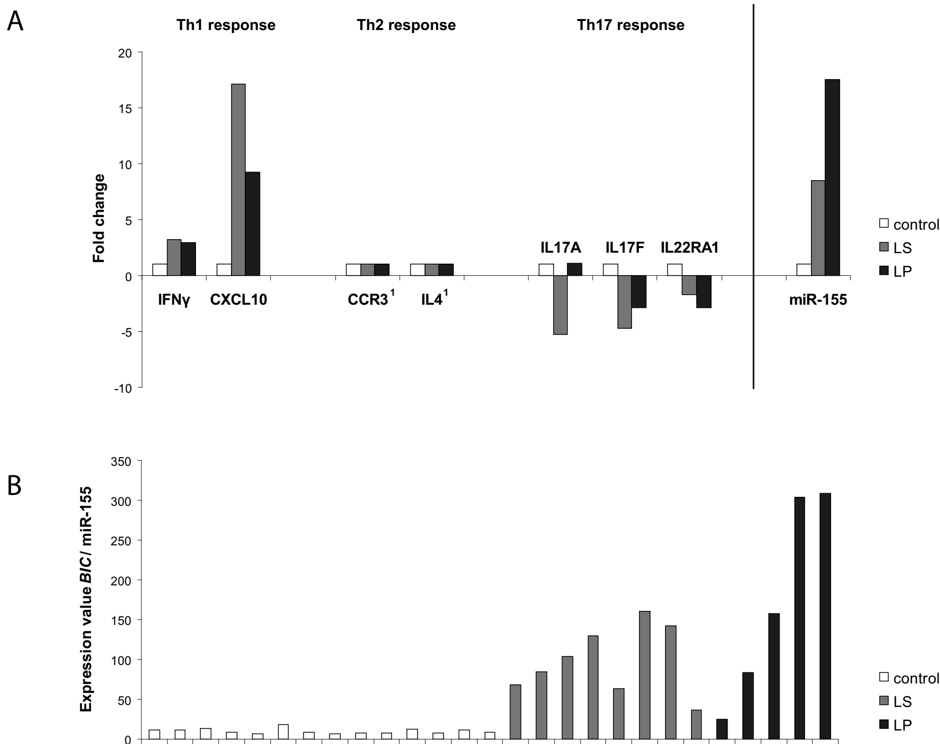

Figure 3. Real-time RT-PCR results. A. Differences in gene expression are visualized for key players in the Th1, Th2 and Th17 response and validation of miR-155 expression by TaqMan RT-PCR (control n=5, lichen sclerosus (LS) n=5 and lichen planus (LP) n=5). [a] No significant difference on microarray data and therefore no real-time RT-PCR was performed. **B.** Gene expression of *BIC*/miR-155 in controls (n=14), LS (n=8) and LP (n=5).

an important role in regulating homeostasis of the immune system. It regulates production of cytokines, chemokines and transcription factors, but has also been shown to promote T cell-dependent autoimmune inflammation.[25-26] We found strong upregulation of *BIC*/miR-155 in LS (9.5-fold) and LP (17.7-fold) compared to controls (Figure 3B). This upregulation was confirmed by TaqMan RT-PCR (LS: 8,5-fold and LP:17.5-fold, Figure 3A).

Immune cell counts in LS and LP

In order to document the composition of the cellular infiltrate, presence of CD4$^+$ T cells, CD8$^+$ T cells, FOXP3$^+$ Treg cells, CD1a$^+$ DCs/Langerhans cells and CD19$^+$ B cells was assessed. In LS no differences in immune cell counts were observed in the epidermis compared to controls. However, in the dermis, cell counts for CD8$^+$ T cells and FOXP3$^+$ Treg cells were significantly higher than in controls ($P < 0.001$ and $P = 0.013$ respectively, Figure 4 and Table 1). In contrast, CD1a$^+$ cells were significantly decreased in the dermis ($P = 0.008$, Table 1).

In the epidermis of LP, FOXP3$^+$ Treg cells were significantly increased compared to controls ($P = 0.024$, Table 1), and no differences were observed for the other immune cells. In the dermis, however, a significant increase in CD4$^+$ T cells, CD8$^+$ T cells,

Table 1A and B. Immunocompetent cell counts (cells/ mm^2) in epidermis and dermis in tissues of healthy controls, patients with lichen sclerosus (LS), and patients with lichen planus (LP).

A	**Controls** (n=10)	**LS** (n=16)	**LP** (n=9)			
Markers	**(1)** median (range)	**(2)** median (range)	**(3)** median (range)	P-value 1 vs **2** vs 3	P-value **1** vs **2**	P-value **1** vs **3**
CD8+	139 (101-437)	326 (70-821)	202 (14-752)	0.15	0.08	0.33
CD4+	236 (93-478)[a]	372 (141-944)	346 (209-743)	0.24	0.10	0.10
FOXP3+	2 (0-29)	12 (0-73)	17 (0-78)	0.029	0.10	0.024
CD19+	0 (0-1)	0 (0-0)[b]	0 (0-4)[c]	0.06	0.22	0.30
CD1a$^+$	255 (82-449)	211 (56-479)	232 (104-1167)[d]	0.57	0.60	0.65

B	**Controls** (n=10)	**LS** (n=16)	**LP** (n=9)			
Markers	**(1)** median range	**(2)** median (range)	**(3)** median (range)	P-value **1** vs **2** vs **3**	P-value **1** vs **2**	P-value **1** vs **3**
CD8+	210 (82-361)	667 (317-2200)	840 (181-2848)	<0.001	<0.001	0.002
CD4+	730 (274-999)[a]	968 (594-2480)	1420 (907-2392)	0.001	0.07	<0.001
FOXP3+	28 (8-77)	63 (4-181)	270 (63-831)	<0.001	0.013	<0.001
CD19+	17 (0-171)	36 (0-709)[b]	147 (84-373)[c]	<0.001	0.12	0.006
CD1a$^+$	53 (16-155)	11 (0-372)	39 (2-329)[d]	0.002	0.008	0.88

CD8$^+$, cytotoxic T-cells; **CD4$^+$**, T-helper cells; **FOXP3$^+$**, Treg cells; **CD19$^+$**, B-cells; **CD1a$^+$**, Langerhans cells. Differences between groups were calculated using the Kruskal-Wallis test, and inter-group differences with the Mann-Whitney test. [a] n=9, [b] n=15, [c] n=7, [d] n=10.

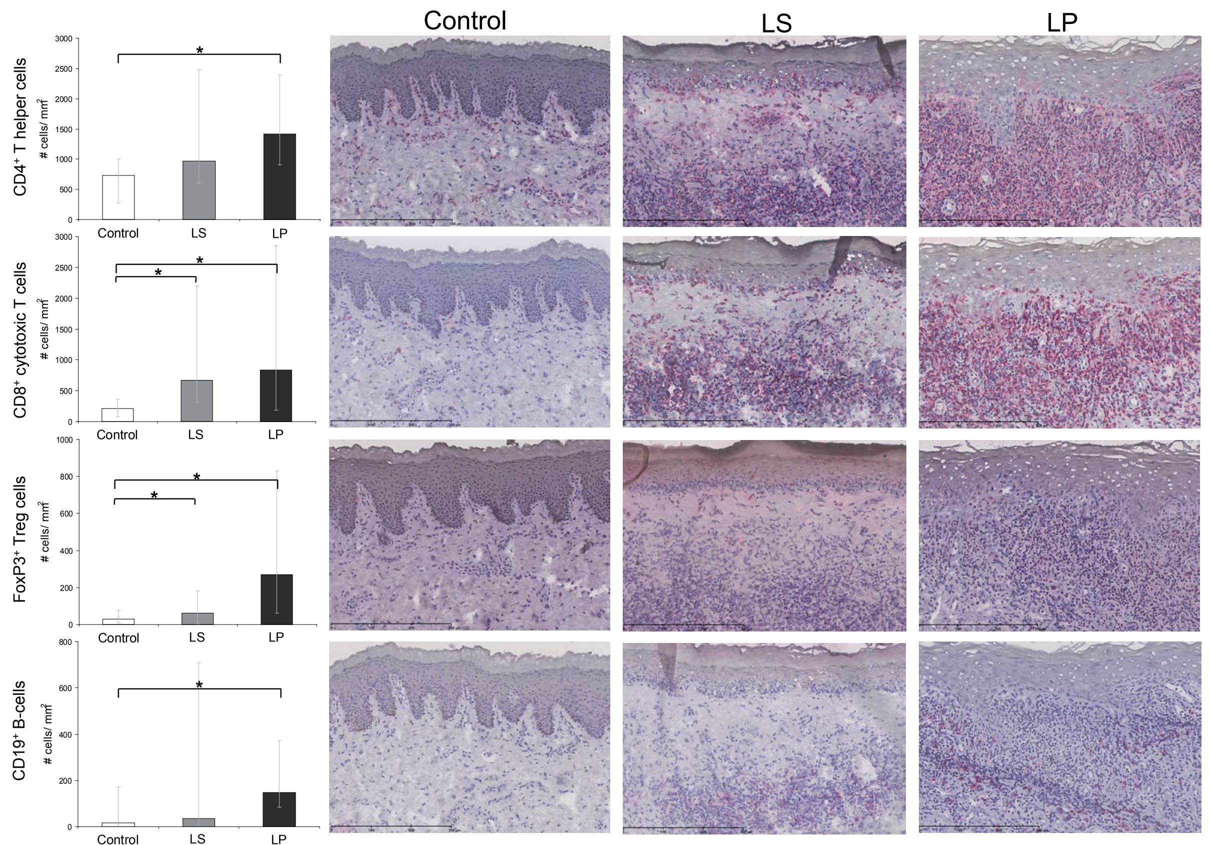

Figure 4. Immune cell counts in lichen sclerosus (LS) and lichen planus (LP). Numbers of immune cells in controls (n=10), LS (n=16) and LP (n=9) and immunohistochemical staining for CD4, CD8, FOXP3 and CD19 (20x magnification). Median ± range. *, $P < 0.05$ (Mann-Whitney test). Bar represents 300μm.

FOXP3+ Treg cells and CD19+ B cells was observed in LP in comparison with controls (Figure 4 and Table 1).

Similar to differences in gene expression levels, differences in immune cell counts were more pronounced in LP than in LS.

Autoantibodies in LS and LP

On basis of our results, and supported by reports in the literature,[12-13,17] LS and LP could very well be autoimmune disorders. As the presence of autoantibodies is characteristic for autoimmune diseases, we retrospectively investigated the presence of autoantibodies in the serum of LS and LP patients. Data on autoantibodies (ANA, anti-TPO and anti-Tg) were available for 106 LP and 42 LS patients. Thirty-four LP patients (32%) and 6 LS patients (14%) were positive for one or more autoantibodies. In LP patients, we observed positive ANA titers in 18/101 patients (18%), positive anti-TPO titers in 11/83 patients (13%) and positive anti-TG titers in 9/71 patients (13%). In LS patients we found positive ANA titers in 5/40 patients (13%). Unfortunately, insufficient data on anti-TPO and anti-Tg were available in LS patients: of only 6 LS patients we found data on TPO antibodies, one of them had a positive titer. No data were available for anti-Tg in LS (Figure 5). In summary, high levels of serum autoantibodies were observed in LS and LP.

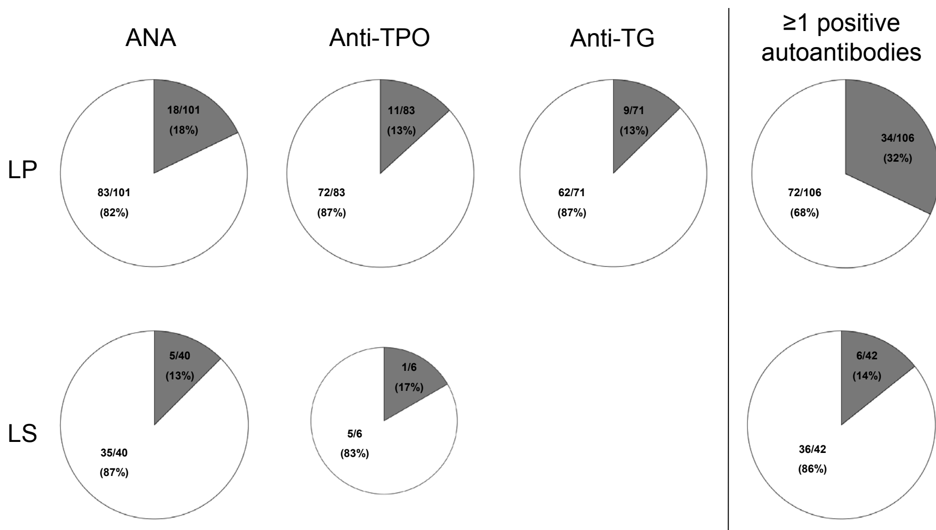

Figure 5. Autoantibodies in lichen sclerosus (LS) and lichen planus (LP) patients. ANA, antinuclear antibodies; antithyroid peroxidase antibodies (anti-TPO); antithyroglobulin antibodies (anti-Tg).

DISCUSSION

Recent publications indicate that LS and LP may be autoimmune disorders. Firstly, like autoimmune diseases, LS and LP are more common in women than in men. Secondly, patients with LS and LP are more frequently diagnosed with other autoimmune disorders such as vitiligo and thyroid disease, and often have high levels of circulating autoantibodies.[17] Also, the presence of autoreactive T cells against basement membrane zone components has been observed in LS and LP.[27-30] Lastly, an association of LS with HLA class II antigen DQ7 and of LP with HLA DRB1*0201 has been demonstrated.[11-13]

From the above, it is hypothesized that aberrant immune signaling plays a role in the pathogenesis of LS and LP, although the exact molecular mechanisms which lead to these immunological changes have not yet been clarified. Here we will discuss our main findings which further corroborate that autoimmunological mechanisms are the basis of both LS and LP.

LS and LP are characterized by a strong Th1 response.

In both disorders, the lymphocytic infiltrate contained primarily T cells. In LS patients, a significant increase in $CD8^+$ T cells and Treg cells was observed, while $CD4^+$ T cell counts in LS seemed to be increased, although this was not significant (P = 0.07). For LP, a more pronounced increase for all immune cell counts was observed, with significantly increased T- and B cell counts. These data are in agreement with published investigations describing dense T cell infiltrates in LS and LP.[31-36]

To investigate whether these T cells are involved in a Th1 or Th2 response, we studied the different chemokine receptors. Th1 cells are characterized by the expression of CXCR3 and CCR5, while Th2 cells express CCR3 and CCR4.[24] We found high levels of *CXCR3* and *CCR5* in LS and LP and no expression of *CCR3* and *CCR4*, indicating an infiltrate of Th1 cells. Although the role of Th1 response in autoimmune disorders is not completely understood, it seems to play an important role in the induction, maintenance and exacerbation of chronic inflammation. This inflammation is controlled by the presence of Th1 cells, which produce IFNγ. High levels of IFNγ will attract and stimulate different immune and epithelial cells. In reaction to this, these cells will produce ligands for the CXCR3 and CCR5 receptors, namely cytokines CXCL9, CXCL10, CXCL11, CCL3 and CCL4 (Figure 2), thereby attracting more Th1 cells to the site of inflammation. As a result the Th1 response will be maintained and intensified, resulting in chronic inflammation.[37]

The role of the above described cytokines in relation to autoimmunity has previously been investigated and reviewed.[37-39] In various autoimmune disorders, like multiple sclerosis,[40-42] rheumatoid arthritis,[43] systemic lupus erythematosus (SLE),[44] Graves' disease,[45-46] type 1 diabetes mellitus[47] and vitiligo,[48] high levels of CXCR3 and its ligands are described (Table 2). In Figure 6 a schematic representation of this feed-forward autoinflammatory process is shown, which, according to our data, plays a major role in the autoimmunological pathogenesis of LS and LP.

Table 2. Expression of cytokines and miR-155 in different autoimmune disorders according to literature.

Autoimmune disorder	Cytokine profile
Multiple Sclerosis[40-42,60]	CXCR3↑, CXCL9↑, CXCL10↑, IFNγ↑, miR-155↑
Rheumatoid arthritis[43,58,61]	CXCR3↑, CXCL9↑, CXCL10↑, IFNγ↑, miR-155↑
Systemic Lupus Erythematosus[44,59]	CXCR3↑, CXCL9↑, CXCL10↑, IFNγ↑, miR-155↑
Graves' disease[45,46]	CXCR3↑, CXCL9↑, CXCL10↑, IFNγ↑
Type 1 diabetes mellitus[47]	CXCR3↑, CXCL9↑, CXCL10↑, IFNγ↑
Vitiligo[48]	CXCR3↑, IFNγ↑

Next to increased immune cell counts and expression of pro-inflammatory cytokines, we also observed an increased expression of miR-155. As indicated in Figure 6, elevated expression of miR-155 may be a primary molecular event that initiates the activation of immune cells. This concept will be discussed in the next section.

MiR-155 is profoundly upregulated in LS and LP

MicroRNAs are small endogenous noncoding RNAs that post-transcriptionally regulate gene expression by base-pairing to imperfect complementary target sites on the RNA, or by partially blocking translation.[49-50] It is known that miR-155 plays a critical role in regulating homeostasis of the immune system. It regulates production of cytokines, chemokines and transcription factors, and is induced by endotoxins via toll-like receptor signaling.[25, 51-53] Furthermore, miR-155 is expressed in several types of activated immune cells, such as macrophages, DCs, B and T cells, and promotes T cell differentiation towards Th1.[25, 51, 54-56]

Based on this, it can be hypothesized that increased expression of miR-155 in LS and LP originates from the dense T cell infiltrate in both disorders. However, we did not observe overexpression of *BIC*/miR-155 in usual type vulvar intraepithelial neoplasia (uVIN), a vulvar disease with also a strong dermal lymphocytic infiltrate (supplementary Figure 2 (http://www.erasmusmc.nl/47393/1584119/1603959/Sante-goets)). A possible explanation for the difference in expression of miR-155 in LS/LP and in uVIN could be that the lymphocytic infiltrate in LS and LP primarily consists of *activated* T cells, while in uVIN the T cell response seems to be ineffective since these T cells are unable to clear HPV, the underlying cause of uVIN.[57]

A role for miR-155 in autoimmune diseases has been demonstrated recently, and increased expression was observed in different autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis (Table 2).[26,58-61] One important mechanism inducing autoimmunity is loss of immune tolerance. Normally, unwanted immune responses are controlled by the suppressive function of Treg cells, which are known to play a critical role in maintenance of immune tolerance. In a mouse model studying SLE, it was observed that artificially enhanced miR-155 expression resulted in an altered Treg cell phenotype and thereby in a reduced suppressive function

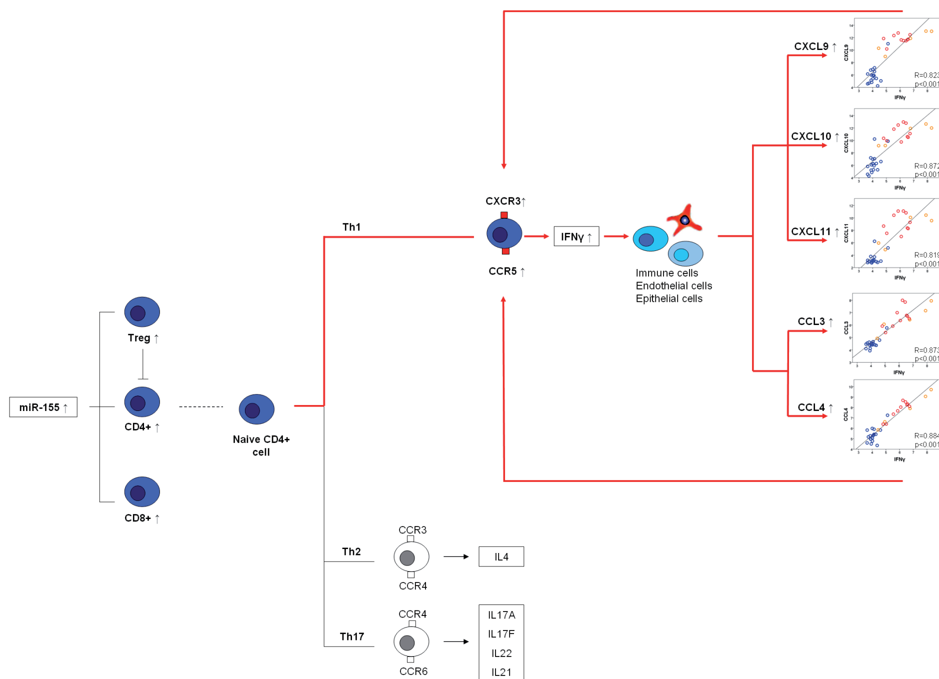

Figure 6. Hypothesized mechanism of pathogenesis of lichen sclerosus (LS) and lichen planus (LP). MiR-155 is strongly upregulated in LS and LP, and possibly affects Treg cell mediated suppression of CD4⁺ T cells by affecting the suppressive function of Treg cells and by reducing the susceptibility of CD4⁺ T cells for Treg cell mediated suppression. The T cell response is skewed to Th1. Upregulation of CXCR3 and CCR5 (Th1 receptors) results in increased production of IFNγ, which attracts different dendritic, epithelial and endothelial cells. In response, these cells will produce high amounts of CXCL9, CXCL10, CXCL11, and CCL3, CCL4 which in turn intensify and maintain the Th 1 response. Correlations between IFNγ and these ligands are shown on the right (Spearman's correlation coefficient). Blue: controls (n=18); red: LS (n=10); orange: LP (n=5).

of Treg cells (despite an increase in Treg cell counts).[59] In addition, another study showed that increased miR-155 levels in CD4⁺ T cells resulted in reduced Treg cell mediated suppression.[62] For LS and LP a similar mechanism may be at work: because of enhanced miR-155 levels the observed increased numbers of Treg cells may not fully affect CD4⁺ T cells thus resulting in impaired immune tolerance towards self-antigens causing autoimmunity.

LS and LP have profound characteristics of an autoimmune disorder

Besides a strong Th1 response and overexpression of miR-155, we also found autoantibodies in a high percentage of patients with LP (32 %) and LS (14 %), although for LS the patient numbers were small. Despite the fact that these data are retrospective, the results are in line with previous data, mostly from small descriptive cohort studies.[63-65] Recently Cooper et al. performed a case-control study in which

they found significant higher levels of auto-antibodies in 52/126 (41%) women with LP and 39/190 (21%) women with LS compared to controls.[17]

Summarizing our data, we demonstrate high levels of *BIC*/miR-155 and increased gene expression of pro-inflammatory cytokines involved in autoimmunity, with an important role for CXCR3 and CCR5 and their ligands resulting in a dense infiltration of T cells. Overall, these observations were more pronounced in LP than in LS. Together with the finding of autoantibodies in a high percentage of patients, and previous data about associations with HLA-DR, other autoimmune diseases and demonstration of autoreactive T cells, an autoimmunological basis for LS and LP seems likely. Future studies should focus on the role of miR-155 and the immune response in these disorders, which may be a focus for more targeted therapies.

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4 DISCUSSION

DISCUSSION

In this thesis molecular and immunological mechanisms involved in the pathogenesis of different vulvar disorders are described. Better knowledge of the molecular changes that occur in these disorders will not only provide a better understanding of the pathogenesis, but may also help to develop new treatment options. Therefore, in the following sections the clinical implications of our findings and future research directions will be discussed.

HPV related epithelial disorders of the vulva

Malignant potential of HPV

Since infection with a high-risk HPV is a well established risk factor for different human cancers[1], it is important to understand the molecular changes that occur during a persistent HPV infection. By examining the molecular profiles of lesions that may progress into cancer, such as usual type VIN (uVIN) as a precursor for vulvar cancer, the involvement of different biological pathways and processes during a persistent HPV infection were evaluated in chapter 2. The malignant potential of uVIN was demonstrated by showing that several hallmarks of cancer were already displayed in this disorder.[2] For example, uVIN is highly proliferative, due to dysregulation of several important genes involved in cell cycle control, such as cyclinA (*CCNA*), cyclinB (*CCNB*) and cyclinE (*CCNE*).[2] These cyclins are important for a cell's progression through the cell cycle.

HPV oncogenes E6 and E7 play an important role in the dysregulation of HPV induced progression to cancer.[3] Enhanced expression of these oncoproteins is caused by integration of the viral DNA into the host's genome, which is considered one of the key events in the pathogenesis of HPV related cancers.[4] Viral integration into DNA is found in many high-grade lesions and cancers, while it is rarely observed in low-grade lesions. This phenomenon has been largely studied in cervical carcinogenesis, where the proportion of samples with integration of HPV 16/18 increases with the severity of cervical lesions.[5-7] For vulvar neoplasia, however, few data are available about HPV integration. Hillemans et al. found integration of HPV 16 or 18 in 8/21 (38.1%) of VIN3 lesions, while no integration was found in VIN 1 and 2 lesions.[8] Van de Nieuwenhof et al. studied integration of HPV 16/18/31/33 in uVIN lesions adjacent to vulvar squamous cell cancer. Integration of viral DNA was observed in 24/25 (96%) of these uVIN lesions.[9]

Integration of the HPV genome results in increased genetic instability, which is illustrated by the fact that most HPV related cancers have numerous chromosomal abnormalities.[3, 10] Consistent with this notion, aneuploidy is a common finding in HPV immortalized cell lines and can also be detected in HPV related premalignant lesions such as Cervical Intraepithelial Neoplasia (CIN) and uVIN.[10] In addition, Van der Avoort et al. studied aneuploidy in non-HPV related differentiated type VIN (dVIN) and vulvar cancer lesions. DNA aneuploidy was found in 38% of dVIN lesions and in

53% of vulvar cancers.[11] These findings illustrate that genetic instability seems to play a role in HPV-dependent as well as HPV-independent vulvar carcinogenesis.

New treatment strategies for HPV related usual type VIN

In chapter 2.2 we describe that the innate as well as the adaptive immune response is disturbed in uVIN lesions.[12] Of special importance for the innate immune response are dendritic cells (DCs). By studying the expression of different chemokines in skin biopsies of uVIN lesions, it seems that a persistent infection with HPV lead to normal maturation of DCs. However, due to lack of accurate chemokine signaling, the process of migration of these mature DCs towards the draining lymph node to activate the adaptive immune response, seems to be diminished. The adaptive immune response consists of a cell-mediated immune response and a humoral immune response. The cell-mediated immune response is characterized by the infiltration of effector T- cells. Dependent on which response is required to eliminate a pathogen, naïve CD4+ T cells will differentiate into type 1 T-helper cells (Th1), type 2 T-helper cells (Th2), type 17 T-helper cells (Th17) or Treg cells. This differentiation is mainly driven by cytokines produced by DCs. Beside this local response, also a systemic Th1 response is required to successfully attack HPV, since a Th1 response can be detected in the majority of healthy sexually active individuals, while it is weak or absent in patients with neoplasia caused by high-risk HPV.[13-14]

Because there is a diminished immune response in uVIN lesions, different therapies to stimulate the immune response have been studied. Imiquimod is an immune stimulator that can bring on a Th1 immune response by induction of secretion of pro-inflammatory cytokines.[15-16] In contrast to surgical procedures, which for long were the standard treatment for uVIN, imiquimod preserves vulvar anatomy and can be administered by the patient herself at home. The effectiveness of imiquimod in uVIN was studied by different groups showing that complete response rates vary from 26 to 100% and partial response rates from 0 to 60%.[17-18] The randomized controlled trial of van Seters et al. showed a complete response rate of 35% and a partial response rate of 46%.[18] Furthermore, a follow-up study (> 5 year) of these patients indicated that imiquimod is effective in the long term, mainly in patients who display a complete response with clearance of HPV after initial treatment.[19] Also, in some patients with a partial response, a complete response was achieved after prolongation of imiquimod treatment. However, taking all these data together, the long term complete response rate after imiquimod treatment is less than 50% which leaves room for alternative treatments.

Recently the effectiveness of vaccination with a synthetic long-peptide vaccine against HPV-16 oncoproteins E6 and E7 was studied.[20] A clinical response and relief of symptoms was observed in 79% of patients, with a complete response in 47%. Interestingly, non- or partial responders showed to have larger lesions on average and these patients displayed a weaker effector T cell response, demonstrated by a significantly lower IFNγ/IL-10 ratio after vaccination.[21] The fact that non- or partial responders have larger lesions was also noted by Terlou et al,[19] and consistent with

this notion it seems crucial to start treatment as early as possible, when lesions are still small.

Another new therapy is photodynamic therapy (PDT), which uses application of the photosensitizer 5-aminolevulinic acid (ALA) in combination with light exposure to induce cell death. As a consequence of massive cell death, a local inflammatory response is induced. The effectiveness of PDT in uVIN was studied by different groups and response rates vary widely from 0 to 71%.[22]

Since the above described therapies never reach response rates close to a 100%, it is hypothesized that combinations of these treatment options (imiquimod, therapeutic vaccination or PDT), can further optimize the immune response and thereby improve response rates. First phase II clinical trials investigating combinations are currently performed.[23-24] Daayana et al. studied the combination of topical imiquimod followed by therapeutic HPV vaccine and found a complete response in 63% of patients. These responders showed to have increased levels of CD4+ and CD8+ T cells, whereas the non-responders had increased levels of regulatory T cells (Treg).[23] Similar results were found in the combination of imiquimod and PDT, where non-responders also demonstrated significantly higher levels of Treg cells.[24] These data suggest that non-responders have an unfavorable local immune environment, with increased levels of Treg cells, resulting in an ineffective IFNγ induced T cell response.

In summary, the following factors seem to be important in the treatment of uVIN. Firstly, an effective IFNγ induced T cell response is necessary to clear the persistent infection with high risk HPV. Secondly, treatment should be started as early as possible, when lesions are small. Thirdly, in partial responders prolongation of therapy may be successful. Which combination of therapies is most optimal or in which order therapies should be provided, however, still remains unclear. Therefore, multicenter randomized trials with standardized outcome measures are needed. Furthermore, it is important to realize that reaching a 100% cure rate will be a serious challenge, demonstrating the importance of the development of alternative new treatment approaches.

Prophylactic HPV vaccination

For uVIN the risk of progression into vulvar cancer is estimated to be 9%.[25] Fortunately, a decrease in incidence of HPV related gynaecological disorders, including uVIN and vulvar cancer, is to be expected with the introduction of the prophylactic HPV vaccine. In 2009, a bivalent vaccine that protects against HPV16 and 18 was introduced in the National Immunisation Program for 12-year-old girls. This vaccine is highly efficient in preventing persistent HPV infections of cervix, vagina, vulva and anus and will hopefully result in a decrease of HPV related gynaecological cancers in the future.[26] Besides this bivalent vaccine, a quadrivalent HPV vaccine is also available. The effectiveness of this vaccine (directed against HPV types 6,11, 16 and 18) on uVIN was recently published from a multinational phase III trial. The efficacy against VIN 2–3 related to HPV 16 and/or 18 was 94.9% in the HPV-naive population and

75.6% in the intention to treat population, where women were previously exposed or unexposed to HPV.[27]

Whether these new vaccination strategies will result in a reduction of HPV related cancer incidence, will depend on various factors, including acceptance of and accessibility to the vaccine, duration of immunity, and cross-protection against other high-risk HPV types. In the meantime, the necessity for screening programs and the development of new, effective treatment strategies remains necessary.

Non-HPV related epithelial disorders of the vulva

New treatment strategies for lichen sclerosus and lichen planus

In chapter 3 we describe several characteristics of two commonly diagnosed chronic dermatological vulvar disorders, namely lichen sclerosus and lichen planus. It seems that both disorders are diagnosed more frequently in recent years, but exact data on the incidence or prevalence rates are lacking. In studies of patients attending vulvar clinics, the prevalence of lichen sclerosus was 34-38 % and of lichen planus 6-11 %.[28-29]

Treatment of first choice in both disorders is application of a topical corticosteroid.[30] However, a large variation in dose and interval of application is described, mainly based on personal experience of the treating physician. The development of uniform guidelines could be helpful in optimization of treatment. Currently, in the Netherlands a multidisciplinary team (consisting of dermatologists, gynaecologists, pathologists, urologists, gastroenterologists, general practioners, sexuologists and dental surgeons) in close collaboration with the patients supportgroups "Stichting Lichen Sclerosus" and "Lichen Planus Vereniging Nederland", is developing such a national guideline.

If treatment with steroids is unsuccessful, the use of topical calcineurin inhibitors (tacrolimus or pimecrolimus) can be considered.[31] Unlike steroids, these immunosuppressive agents do not cause atrophy. However, since the Food and Drug Administration issued a warning for these drugs due to the lack of long-term safety data and the potential risk of development of malignancies, they nowadays are only prescribed as a second-line treatment for short-term and intermittent use.[32]

Although potent corticosteroids and calcineurin inhibitors do provide significant relief of symptoms, there is a lack of clinical trials providing evidence to what extent these immunosuppressive treatment modalities actually work. Only a few case reports are described and the few randomized clinical trials that are performed have small sample sizes. Furthermore, none of the immunosuppressive therapies is curative, illustrating the importance of development of new treatment strategies.

As we found that chemokine receptor 3 (CXCR3) is highly upregulated in both lichen sclerosus and lichen planus (chapter 3.2), it can be hypothesized that inhibition of its production can be such a new therapeutic option. Because overexpression of CXCR3 is also observed in different autoimmune disorders, such as rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus and Graves' disease, the pharmaceutical industry invests in the development of drugs that inhibit or block the CXCR3 receptor.[33-35] Until now several small, nonpeptidergic antagonists have been found, most of them studied in experimental/preclinical settings.[36] One of them was

AMG487, studied in animal models but also in a Phase IIa clinical trial on psoriasis. Unfortunately no effect was observed and the trial has been halted.[36] This illustrates the complexity of the development of drugs that block chemokine activity, since chemokines and their receptors have pleiotropic and sometimes opposing biological effects. Simply blocking the CXCR3 receptor by an antagonist may result in unwanted effects, like inhibition of the inflammatory process. Therefore, it is still debatable whether or not these antagonists will be successful in the treatment of autoimmune disorders and accordingly in the treatment of lichen sclerosus and lichen planus.

Another interesting finding was the high expression of miR-155 in lichen sclerosus (9.5 fold change) and lichen planus (17.7 fold change) (Chapter 3.2). MiR-155 plays an important role in regulation of the immune response and it is expressed in different immune cells.[37-39] High expression of miR-155 is also observed in different auto-immune disorders, such as multiple sclerosis and rheumatoid arthritis.[40-41] On the basis of these findings it is speculated that manipulation of miR-155 may be effective in the treatment of these disorders.[42-44] Although we believe that miR-155 plays a role in the pathogenesis of lichen sclerosus and lichen planus, its exact role needs to be elucidated. Therefore, it would be of interest to study which cells express miR-155 and how miR-155 exactly influences the immune response to induce autoimmunity. Improving our understanding of miR-155 in lichen sclerosus and lichen planus, may help to develop new treatment strategies for both disorders.

Malignant potential of lichen sclerosus and lichen planus

During follow-up of patients with lichen sclerosus and lichen planus, vulvar malignancies may be detected.[30] Therefore lifelong follow-up is advised and all suspicious lesions should be biopsied in order to rule out dVIN or invasion. Unfortunately, histological evaluation of these biopsies by pathologists is often a challenge. This is mainly due to the fact that dVIN can be easily mistaken for a benign dermatosis because of the high degree of cellular differentiation and absence of widespread architectural disarray.[22] Furthermore, the histological evaluation of lichen sclerosus and lichen planus can also be difficult, because especially the first phase of lichen sclerosus can show histological overlap with lichen planus.[45] Ultimately, the development of specific markers to distinguish both diseases from each other should be a helpful diagnostic tool. This is a challenge, since we found almost no differences between the gene expression profiles of both disorders. Only 623 genes were observed to be differentially expressed between lichen sclerosus and lichen planus and none of these differentially expressed genes was exclusively regulated in one of the disorders. (Chapter 3.2)

In contrast, we do have markers that distinguish between the HPV related and the non-HPV related pathway towards vulvar cancer. Hoevenaars et al. described a specific immunohistochemical profile for those two pathways. In the HPV related pathway, uVIN lesions showed high expression of nuclear p16^{INK4A}, while no expression was observed in dVIN lesions involved in the non-HPV related pathway. On the other hand, p53 staining was clearly enhanced in the basal cell layer of dVIN lesions, while

in uVIN occasionally a distinct clustered pattern was observed in central parts of the rete ridges. Furthermore, Hoevenaars et al. described a difference in the localization of MIB1 staining. uVIN lesions showed MIB1 expression throughout the whole epidermis, while in dVIN lesions MIB1 expression was limited to the lower layers of the epidermis.[46]

In conclusion, both pathways leading to vulvar cancer can be characterized by an unique immunohistochemical profile: $p16^{INK4A}$ staining for the HPV related pathway and p53 staining for the non-HPV related pathway. Until now, however, no prognostic markers are available and since the exact mechanisms of progression of lichen planus and lichen sclerosus into dVIN and further into vulvar cancer remain unclear, further research in this field is necessary.

Increasing awareness of vulvar disorders

It seems that vulvar disorders have been underdiagnosed for a long period of time, due to both doctors' and patients' delay. Doctors' delay is mainly caused by a lack of knowledge about vulvar disorders among general practioners, but also among gynecologists and dermatologists. As a result, women often have a history of long lasting symptoms, before an accurate diagnosis is made. For example, in our clinical study about genital lichen planus described in chapter 3.1, 19% of women had symptoms for more than one year, 25% of women had symptoms between 2 and 5 years and in 34% symptoms were even present for more than 5 years.[47] Patients' delay, on the other hand is also a significant factor in underdiagnosis, and is primarily caused by the fact that vulvar complaints are still a taboo.

To increase patients' and doctors' awareness, promotion of specialized vulvar clinics should be encouraged. The introduction of internet pages as www.vulvapoli.nl and the founding of professional organizations as the International Society for the Study of Vulvovaginal Disease (ISSVD), the "Nederlandse Vereniging voor Vulva Pathologie" (NVvVP), and the foundation of patients' supportgroups such as "Stichting Lichen Sclerosus" and "Lichen Planus Vereniging Nederland", are important in this context. At this moment about 30 vulvar clinics are active in the Netherlands. It can be expected that more clinics will be opened in the near future, as the demand for specialized vulvar care is increasing. The introduction of these vulvar clinics can be a helpful teaching and training opportunity for clinicians, but is also important for the implementation of research and clinical trails on vulvar disorders.

Conclusion

The studies presented in this thesis demonstrated several molecular and immunological mechanisms in HPV related and non-HPV related epithelial disorders of the vulva. These new insights can help to improve existing, or develop new treatment strategies as described in this chapter. Furthermore, we underlined the importance of specialized care for patients with vulvar disorders to improve patient care, to facilitate teaching and training opportunities, to generate new research questions and to initiate new studies.

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5 SUMMARY SAMENVATTING

SUMMARY

The vulva is the outer part of the female genital tract, consisting of the mons pubis, the labia majora and minora, the clitoris, the vestibule of the vagina and the urethral orifice. Different disorders can affect the vulva and may have a considerable influence on quality of live and sexual functioning. Treatment of these disorders is often difficult and unsatisfying. In addition, since some of these disorders can result in vulvar cancer, lifelong follow-up is advised. Therefore, treatment of patients with vulvar disorders should preferably take place in specialized vulvar clinics, where dermatologists and gynaecologists work together.

Basic knowledge about vulvar disorders is sparse. Therefore, the aim of this thesis was to gain more insight in the molecular and immunological mechanisms of different epithelial disorders of the vulva. This in order to provide a better understanding of the pathogenesis, and eventually provide new insights for development of new treatment strategies.

Chapter 1 is a general introduction. Two groups of vulvar disorders are introduced. First, the disorders caused by a persistent infection with human papillomavirus (HPV), namely condylomata acuminata, better known as genital warts, and usual type Vulvar Intraepithelial Neoplasia (uVIN). Second, two common chronic dermatological vulvar disorders are introduced: lichen sclerosus and lichen planus. Furthermore, because immunity is important in the pathogenesis of these disorders, the role of the immune system is explained.

In **chapter 2.1** the molecular characteristics of uVIN are described. uVIN is caused by a persistent infection with a high-risk HPV type. Although it is still a premalignant disorder, we observed that uVIN already displays several hallmarks of cancer. For example, it appears to be a highly proliferative disease: many cyclins, which are important for regulation of the cell cycle, are upregulated. Furthermore, this proliferation seems to be independent of paracrine or endocrine signals: many receptors (for example the estrogen and androgen receptor) and their ligands are downregulated.

Based on the fact that uVIN is more frequently seen in immune-suppressed women and that the immunostimulator imiquimod is an effective treatment, the host immune response seems of critical importance in determining progression or regression of uVIN. In **chapter 2.2** we studied the immune response in more depth by exploring expression levels of chemokines in relation to the presence or absence of different immune cells. We observed dysregulation of several important chemokines and an increased number of dendritic cells in the dermis in uVIN. Furthermore a significant increase in CD4+ T helper cells and CD8+ cytotoxic T cells was observed. Summarizing these results, our data indicate that in uVIN the innate immune response is inadequate, due to the fact that most dendritic cells do not receive the proper chemokine signal to migrate towards the lymph node. As a result dendritic cells are not able to present the HPV antigen to naïve T cells, resulting in an insufficient

initiation of the adaptive immune response. Consequently, HPV will not be cleared and may cause uVIN.

Persistent HPV infections are a well-established risk factor for a large spectrum of epithelial lesions, ranging from benign hyperplasia, caused by low-risk HPV types (for example HPV type 6 and 11), to (pre)malignant lesions caused by high-risk HPV types (for example HPV type 16 and 18). In the vulva, infection with a low-risk HPV type can result in condylomata acuminata, better known as genital warts, whereas infection with a high-risk HPV type can cause uVIN. In **chapter 2.3** the molecular basis of this difference in malignant potential is investigated by using gene expression profiles of uVIN and condylomata acuminata. We found that tissues infected with high-risk HPV showed more proliferation and displayed more DNA damage than tissues infected with low-risk HPV. Furthermore we demonstrated that $p16^{INK4a}$ is an excellent molecular marker to detect a high-risk HPV infection.

In **chapter 3.1** we evaluated clinical features, histopathology results, treatment regime and follow-up in 95 women with lichen planus. All women were symptomatic, most often complaining of vulvar soreness and burning. In 81.1% sharply demarcated erythematous lesions were found, mostly located at the vestibule. Furthermore 56.8% of women had oral lesions, illustrating the importance of the examination of the oral cavity in patients with suspected genital lichen planus. Treatment of first-choice was a potent topical corticosteroid. During follow-up, 2 women developed vulvar squamous cell cancer, indicating that strict follow-up on a long-term basis is necessary and that all atypical lesion should be biopsied.

Lichen sclerosus and lichen planus are both chronic dermatological disorders. Although autoimmunity has been suggested, the exact pathogenesis of these disorders is still unknown. Therefore, in **chapter 3.2** we investigated different molecular and immunological mechanisms in both disorders. We found increased expression of pro-inflammatory cytokines, characteristic for a Th1 IFNγ induced immune response, and a dense infiltrate of T cells. Furthermore high levels of microRNA-155, a microRNA involved in regulation of the immune response, were found in lichen sclerosus and lichen planus. One of the characteristics of an autoimmune disorder is the presence of auto-antibodies. We also observed high levels of autoantibodies in 34/106 (32%) lichen planus and in 6/42 (14%) lichen sclerosus patients. Together, these results demonstrate an autoimmunological basis for lichen sclerosus and lichen planus.

Chapter 4 provides a general discussion, where the significance of our findings, implications for clinical practice and future research perspectives are discussed.

SAMENVATTING

De vulva is het uitwendige deel van het vrouwelijke geslachtsorgaan en bestaat uit de venusheuvel (mons pubis), de binnenste en buitenste schaamlippen, de clitoris, de toegang tot de vagina (het vestibulum) en de uitgang van de plasbuis (de urethra). Verschillende aandoeningen kunnen voorkomen op de vulva. Deze hebben vaak grote invloed op de kwaliteit van leven en het seksuele functioneren. De behandeling van deze aandoeningen is vaak moeilijk en teleurstellend. Aangezien sommige van deze aandoeningen kunnen leiden tot vulvakanker (schaamlipkanker), wordt levenslange follow-up geadviseerd. Om deze redenen, dienen controles idealiter plaats te vinden op gespecialiseerde vulvapoli's, waar dermatologen en gynaecologen samenwerken.

Basale kennis over aandoeningen van de vulva is schaars. Het doel van dit proefschrift is dan ook meer inzicht te krijgen in de moleculaire en immunologische mechanismen van verschillende epitheliale aandoeningen van de vulva. Op deze manier wordt meer kennis verkregen over de pathogenese, hetgeen in de toekomst kan leiden tot de ontwikkeling van nieuwe behandelingen.

Hoofdstuk 1 is een algemene introductie. Twee groepen van vulva aandoeningen worden beschreven. Allereerst de aandoeningen die veroorzaakt worden door een persisterende infectie met het humane papillomavirus (HPV), namelijk genitale wratten en *usual type* Vulvaire Intraepitheliale Neoplasie (uVIN). Ten tweede wordt een introductie gegeven over twee regelmatig voorkomende chronische dermatologische vulva aandoeningen: lichen sclerosus en lichen planus. Omdat het afweersysteem een belangrijke rol speelt bij het ontstaan van bovenstaande aandoeningen, wordt daarnaast de werking van het afweersysteem beschreven.

In **hoofdstuk 2.1** worden de moleculaire karakteristieken van uVIN beschreven. uVIN wordt veroorzaakt door een persisterende infectie met een hoog risico HPV-type. uVIN wordt gezien als een premaligne afwijking. Echter, in deze studie tonen we met behulp van genexpressie profielen aan dat het ziektebeeld reeds een aantal maligne kenmerken vertoont. Het is een sterk proliferatieve aandoening: veel cyclines, belangrijk in de regulatie van de celcyclus, komen verhoogd tot expressie. Daarnaast lijkt proliferatie onafhankelijk te zijn van paracriene en endocriene signalen; veel receptoren (zoals de oestrogeen en de androgeen receptor) en hun liganden komen verminderd tot expressie.

Aangezien uVIN vaker wordt gezien bij immuungecompromitteerde vrouwen en omdat de immunostimulator imiquimod een effectieve behandeling is gebleken, lijkt het afweersysteem een belangrijke rol te spelen in de progressie of regressie van uVIN. In **hoofdstuk 2.2** bestuderen we het afweersysteem in meer detail, door te kijken naar de expressie van chemokines in relatie met de aan- of afwezigheid van verschillende typen immuuncellen. We vonden dat er in uVIN sprake is van dysregulatie van verschillende belangrijke chemokines en dat er een verhoogd aantal dendritische cellen in de dermis aanwezig is. Daarnaast vonden we een significant

verhoogd aantal CD4+ T helper cellen en CD8+ cytotoxische T cellen. Samenvattend lijkt het dat de *innate* (aangeboren) afweer inadequaat werkt, aangezien de meeste dendritische cellen niet het juiste chemokine signaal krijgen om te migreren naar een lymfklier. Daardoor zijn zij niet in staat het HPV-antigeen te presenteren aan naïve T cellen, hetgeen leidt tot onvoldoende initiatie van de *adaptive* (verworven) afweer. Op deze manier, kan de HPV infectie niet geklaard worden, wat kan leiden tot de ontwikkeling van uVIN.

Persisterende HPV infecties zijn een bewezen risico factor voor verschillende epitheliale afwijkingen, variërend van benigne afwijkingen, veroorzaakt door laag-risico HPV-typen (bijvoorbeeld HPV type 6 en 11), tot (pre)maligne afwijkingen, veroorzaakt door hoog-risico HPV-typen (bijvoorbeeld HPV type 16 en 18). In de vulva kan een infectie met een laag-risico HPV type leiden tot condylomata acuminata, beter bekend als genitale wratten, terwijl een infectie met een hoog-risico HPV typen kan leiden tot uVIN. In **hoofdstuk 2.3** wordt de moleculaire basis voor het verschil in maligne potentieel tussen een infectie met laag-risico en hoog-risico HPV onderzocht door te kijken naar verschillen in genexpressie tussen uVIN en condylomata acuminata . We vonden dat weefsels geïnfecteerd met een hoog-risico HPV meer proliferatie en DNA-schade vertoonden, dan weefsels geïnfecteerd met een laag-risico HPV. Daarnaast toonden we aan dat $p16^{INK4a}$ een uitstekende moleculaire marker is voor de detectie van een hoog-risico HPV infectie.

In **hoofdstuk 3.1** beschrijven we de klinische eigenschappen, histopathologische resultaten, behandelopties en de follow-up van 95 vrouwen met lichen planus. Alle vrouwen waren symptomatisch, met vulvaire pijn en branderigheid als belangrijkste klachten. In 81.1% vonden we scherp begrensde, erythemateuze afwijkingen, veelal ter plaatse van het vestibulum. Daarnaast had 56.8% van de vrouwen afwijkingen in de mond. Dit laatste maakt duidelijk dat het belangrijk is om bij vrouwen met een verdenking op genitale lichen planus tevens de mondholte te onderzoeken. Eerste keus van behandeling was een potente corticosteroid crème. Gedurende de follow-up ontwikkelden 2 vrouwen plaveiselcel kanker van de vulva, hetgeen het belang van strikte follow-up en het belang van biopteren van iedere atypische afwijking demonstreert.

Lichen sclerosus en lichen planus zijn chronische, dermatologische ziektebeelden. Autoimmuniteit wordt gezien als mogelijke oorzaak, echter de exacte pathogenese van deze aandoeningen is tot op heden onbekend. Daarom hebben we in **hoofdstuk 3.2** verschillende moleculaire en immunologische mechanismen van deze aandoeningen onderzocht. We vonden verhoogde expressie van pro-inflammatoire cytokines -welke karakteristiek zijn voor een Th1 IFNγ gereguleerde immuun respons- en een omvangrijk infiltraat van T cellen. Daarnaast vonden we verhoogde expressie van microRNA-155 (een microRNA belangrijk voor de regulering van de afweer) in lichen sclerosus en lichen planus. Een van de karakteristieken van een auto-immuunziekte is de aanwezigheid van auto-antilichamen. Wij vonden auto-antilichamen in 34/106 (32%) van de lichen planus en in 6/42 (14%) van de lichen sclerosus patiënten. Deze resultaten demonstreren dat auto-immunologische mechanismen belangrijk zijn bij het ontstaan van lichen sclerosus en lichen planus.

Hoofdstuk 4 is een algemene beschouwing, waarin de gevonden resultaten, de klinische relevantie en toekomstige studiemogelijkheden worden bediscussieerd.

LIST OF ABBREVIATIONS

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ANA	antinuclear antibody
ANOVA	analysis of variance
Anti-Tg	antithyroglobulin antibody
Anti-TPO	antithyroid peroxidase antibody
APC	antigen presenting cell
CC	cervical carcinoma
cDNA	complementary DNA
CIN	cervical intraepithelial neoplasia
DAPI	4',6-diamidino-2-phenylindole
DCs	dendritic cells
DNA	deoxyribonucleic acid
dVIN	differentiated type Vulvar Intraepithelial Neoplasia
FA	fanconi anemia
FC	fold change
FDR	false discovery rate
HIV	human immunodeficiency virus
HNSCC	head and neck squamous cell carcinoma
HPV	human papilloma virus
IFN	interferon
IL	interleukin
ISSVD	International Society for the Study of Vulvovaginal Disease
LC	langerhans cell
LP	lichen planus
LS	lichen sclerosus
MHC	major histocompatibility complex
miR	microRNA
mRNA	messenger RNA
NK	natural killer
NO	nitric oxide
NVvVP	Nederlandse Vereniging voor Vulva Pathologie
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PDT	photodynamic therapy
RCT	randomized controlled trial
RNA	ribonucleic acid
RT-PCR	real-time polymerase chain reaction
SAM	significant analysis of microarrays
SCC	squamous cell carcinoma
SEM	standard error of the mean
SLE	systemic lupus erythematosus
Th	T helper
TLR	Toll like receptor
TNF	tumor necrosis factor
Treg	regulatory T-cells
TUNEL	terminal deoxynucleotidyl transferase dUTP nick and labelling
uVIN	usual type Vulvar Intraepithelial Neoplasia
VaIN	vaginal intraepithelial neoplasia
VIN	vulvar intraepithelial neoplasia

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Eindrapport SER jongerenpanel 2007 (including **LAM Santegoets**). Maatschappelijke stages: een schat aan ervaringen.

PHD PORTFOLIO

PHD PORTFOLIO

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» Vulvar Pathology	2006
» Facing a new era of cervical cancer prevention	2008
» Workshop on basic data analysis on gene expression array	2009
» MGC Special course (Leiden) Analysis of microarray gene expression data	2009
» Introduction to data analysis (Erasmus summer program)	2009
» Portfolio course, "Modernisering Medische Vervolgopleidingen"	2009
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National oral presentations	
» Attending and presenting weekly research meeting	2005-2011
» Attending and presenting SGGO meeting	2005-2011
» JNI scientific meeting yearly presentation	2005-2011
» Wetenschapsdag Erasmus MC, Rotterdam	2007
» HPV Journal club, VU Medical Center, Amsterdam	2008
» Molecular medicine Day, Rotterdam	2008
International presentations	
» International Union Against Sexually Transmitted Infections (IUSTI), 23rd Conference, Dubrovnic, Croatia. Oral presentation.	2007
» 15th International Meeting of the European Society of Gynaecological Oncology (ESGO), Berlin, Germany. Oral presentation	2007
» International Gynaecology Cancer Society (IGCS), Bangkok, Thailand. Poster presentation	2008
» ISSVD, Edinburgh, Schotland. Oral presentation	2009

&

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- VO (practical course). Postmenopausaal Bloedverlies 2005-2011
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ABOUT THE AUTHOR

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Lindy Santegoets was born on December 31st 1979 in Goirle, the Netherlands. She grew up in Tilburg and went to secondary school at the Mill Hill College (Gymnasium) in Goirle. In 1999 she started medical school at the Erasmus University in Rotterdam. After her graduation, she started working as MD and PhD student in the department of Obstetrics & Gynaecology (division Oncological Gynaecology) under supervision of prof. dr. Th.J.M. Helmerhorst, dr.ir. L.J. Blok and dr.W.I. van der Meijden. Here, she started the specialized vulvar clinic at the outpatients' clinic of the department.

During her PhD project, in the autumn of 2006, she contributed to "De Nationale DenkTank". This multidisciplinary thinktank has performed research on how to improve quality and efficiency of health care in the Netherlands. The results of this analysis are formulated in ten original recommendations, published as "Recept voor morgen: een frisse blik op betere zorg voor chronisch zieken". After participating in "de Nationale DenkTank", she became a board member of this non-profit foundation. The aim of this foundation is to create a network of people and organizations spanning the main pillars of the Dutch knowledge society: Government, Business and Industry, and Science. Furthermore, she was involved in the foundation of "De Jonge Maatschappij" of "De Koninklijke Hollandsche Maatschappij der Wetenschappen" in Haarlem. In 2007, she was an independent Crown Member of the youth committee at the "Sociaal Economische Raad (SER)". This committee has written an advisory report for the Dutch Government on the topic socio-economic internships.

Since she was selected for an 'AGIKO' (=MD-clinical research trainee) grant from ZonMW, the Netherlands Organization for Health Research and Development, she combined her PhD project with her clinical training; from September 2008 till March 2009 she worked as a resident at the department of Obstetrics & Gynaecology at the Maasstad Ziekenhuis in Rotterdam (Dr. A.M. van Heusden/ Dr. A. Verhoeff) and from October 2009 till October 2010 at the Reinier de Graaf Groep in Delft (Dr. W.A. ter Harmsel/ Dr. H.A. Bremer). After the defense of her thesis (October 2011), she will continue her clinical training in Obstetrics & Gynaecology at the Reinier de Graaf Groep in Delft and at the Erasmus University Medical Center in Rotterdam (Prof.dr. C.W. Burger).

DANKWOORD

DANKWOORD

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Na eerst een jaar in Utrecht gestudeerd te hebben, kon ik in 1999 starten met geneeskunde in Rotterdam. Ik weet nog goed hoe ik er tegen op zag om naar deze stad te verhuizen. Echter al snel bleek dat ik me ook hier thuis kon voelen en in wat voor een huis! Dank lieve Sacha, Marleen, Juul, Merel, Olga en Marieke, jullie waren fantastische huisgenoten. En ook al wonen we niet meer onder één dak, onze vriendschap is uniek. Ik ben blij met jullie! Hoop dat we nog vele jaren samen met onze mannen leuke dingen blijven doen.

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En toen begon het serieuze werk. Wat begon als een afstudeerproject, eindigde in dit promotietraject. Veel dank ben ik dan ook verschuldigd aan mijn driekoppige begeleidingsteam.

Allereerst professor Helmerhorst, mijn promotor. Dank voor uw vertrouwen en enthousiasme. Ik bewonder u manier van leiding geven. Ik ben benieuwd naar uw afscheidsrede in december en ik wens u een fijn emeritaat.

Beste Leen, ik had me geen betere dagelijkse begeleider kunnen wensen dan jij! Jij bewaakte de voortgang en stimuleerde mijn onderzoeksgeest. Daarnaast had je ook interesse voor mijn leven buiten het ziekenhuis. Dank, dank, dank!

Dr. van der Meijden, beste Wim, samen met u begon ik mijn afstudeerproject. Dank voor alles wat u me leerde over de vulvapathologie. Ik vind het fijn dat u vandaag mijn co-promotor bent!

Naast jullie vertrouwen in mijn onderzoek, ondersteunden jullie ook mijn deelname aan de Nationale Denktank. Een super ervaring! Drie maanden lang werd ik ondergedompeld in de wereld van de consultancy met 25 totaal verschillende mensen. Een leerzamer iets, kun je je bijna niet voorstellen. Er ontstonden vriendschappen voor het leven. Lars, onze komiek, wanneer ga je weer voor me zingen? David: als enige vrouw op jouw bachelor, jij met je killing-eyes. Wanneer doen we weer eens een wedstrijdje? Sanne, lief vriendinnetje, hoe bijzonder is het dat jij en Jesse de eerste echte DT-baby hebben. Ons winkeltje, de maandag-bankhang-avondjes, op kraamvistie in Singapore…ik ben blij met jou! Tom, tommyboy: het wordt tijd dat we gaan beginnen aan ons boek. Genoeg avonturen samen beleefd, lijkt me. Zullen we de boom van Clingendael op de kaft zetten? Dank ook voor alle leuke mensen die ik door jou heb leren kennen (Pierre, Marnix en de rest)

Ik vind het weer tijd om samen een weekendje te rellen op een Waddeneiland. Jullie?

Sam en Paul: samen vormen we de drie musketiers. Dank voor al die uren dat jullie mij van mijn werk hebben gehouden. Dit boekje had veel eerder af kunnen zijn, als ik niet altijd onmiddellijk moest 'cummen' om 'noffie' bij Bep te drinken. Of weer eens gebroken achter mijn bureau zat na een avondje aap/skihut of weekendje weg (met de starslet naar villa Lotti / Cumbridge)… maar laat het duidelijk zijn kanjers: dit alles had ik voor geen goud willen missen! Dank jullie wel!

Kamer Hs-508, mijn uitvalsbasis. Al veranderde de samenstelling afgelopen jaren regelmatig, de sfeer bleef goed! Het begon met Emilie, Sharon, Olivier en Durk, en nu eindig ik met Yvonne, Mariëlle en Wendy. Dank voor jullie luisterend oor en de gezelligheid. Annelinde, een speciaal woord van dank aan jou. Samenwerken met jou was top! Zullen we over een aantal jaar samen een vulvapoli beginnen? Jij als dermatoloog en ik als gynaecoloog? Succes met je opleiding en geniet van je leventje in Amsterdam!

Alle andere onderzoekers van de gynaecologie/obstetrie: leuk dat er weer zoveel geborreld word! Succes allemaal met jullie eigen onderzoek en carrière! Passen jullie wel een beetje op John als ik weer in Delft begin? Hij houdt van de gewone koffie van Bep en 1x per week lunchen bij Schmidt Zeevis doet het ook goed. En mocht je hem mee uit willen nemen, geef dit dan tijdig aan in verband met zijn schoenkeuze.

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Onderzoek doen, doe je niet alleen. Graag wil ik dan ook mijn mede-auteurs hartelijk bedanken voor hun input. In het bijzonder wil ik de "vulva-groep" bedanken met daarin Beth Morrel, Patricia Ewing, Marc van Beurden, Manon van Seters, Ilse Beckmann en Alex Kleinjan.

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Aangezien ik als AGIKO, mijn onderzoekstijd moest combineren met opleidingstijd in de kliniek, wil ik ook graag al mijn 'klinische' collega's bedanken! Mijn eerste klinische stappen binnen de gynaecologie zette ik in het Maasstad ziekenhuis onder leiding van Dr. van Heusden. Daarna volbracht ik met zeer veel plezier mijn eerste échte opleidingsjaar in het Reinier de Graaf Gasthuis, met dr. ter Harmsel en dr. Bremer als opleiders en dr. Siemens als tutor. Het is fijn om te weten dat ik weer in dit warme nest terugkom om mijn opleiding af te maken. Ik heb er zin in!

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Victoria, de jaren die ik je ken zijn nog op 1 hand te tellen, maar onze (zondagmiddag)aap-uren allang niet meer…laten we proosten op nog veel meer vriendschapsjaren!

Jennie, nog maar kort in mijn leven, maar wel de moeder van mijn nichtje. Zorg goed voor haar en natuurlijk voor mijn broertje. Ik wens jullie samen alle liefde en geluk.

De leden van de kleine commissie: prof. Burger (tevens mijn dank aan u als hoofdopleider), prof. Bosman en prof. Kenter, wil ik hartelijk bedanken voor de beoordeling van het manuscript.

Mijn paranimfen: Sam en Elske. Jullie zijn beide al genoemd in dit dankwoord, maar weet dat ik het fijn vind dat jullie straks naast mij staan.

En dan als laatste, Evert. Als ik me strikt aan de chronologie had gehouden, had ik je al moeten introduceren tussen alinea 3 en 4. Dit illustreert maar weer hoeveel wij al samen meegemaakt hebben en hoeveel wij hopelijk nog samen mee gaan maken. Ik houd van je!