Taking a closer look: non-invasive tools for in-depth characterisation of vulvar diseases

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Put on your red shoes
and dance the blues

CHAPTER 1

Introduction

The vulva is the name of the external female genital organs delineated by the mons pubis, groin and anus. Individual components of the vulva include the mons pubis, labia majora, labia minora, clitoris, urethral meatus, vaginal vestibulum, vaginal introitus, Bartholin's and Skene's glands and perineum (*Figure 1*).[1] The vulva is a unique anatomical site, functioning as a transition zone from the mucosa of the vagina to cutaneous tissue. Histologically, the vulva is a transitional epithelium. The mons pubis, groin and labia majora display a hirsute, keratinised epithelium, whilst a non-adnexal, nonkeratinized mucosal epithelium with sebaceous glands can be found on the labia minora.[2] Vulvar skin requires resilience against urine, faecal matter and vaginal fluor in addition to pressure and friction from physical activities. Simultaneously, it has an essential role to play in sexual stimulation, intercourse and satisfaction. The vulva also endures the stress of childbirth. Considering the various purposes of the vulva, diseases affecting the vulvar area are often experienced as a serious burden.[3–5] Vulvar disease can consequently affect every aspect of daily life, such as urination, defecation, mobility, sexuality, and clothing choice, resulting in considerable physical and psychological morbidity.[6] Shame, social stigma, and taboo associate strongly with genital diseases, resulting in severe underreporting, lack of clinical recognition, delays in proper treatment, deficient etiological knowledge and paucity in development of novel treatments.[6,7] The spectrum of vulvar diseases encompasses benign and malignant conditions. The subsequent sections will expound upon a selection of vulvar pathologies in greater detail.

PRECURSOR LESIONS OF VSCC: VULVAR HSIL AND DIFFERENTIATED VIN

Vulvar squamous cell carcinoma (VSCC) can arise from two etiologically distinct precursor diseases. The first, vulvar high-grade squamous intraepithelial lesions (HSIL), is caused by human-papillomavirus (HPV) and causes ±20% of VSCC. The remaining ±80% of VSCC cases is preceded by differentiated vulvar intraepithelial neoplasia (dVIN), which develops independently from HPV (*Figure 2*).[8] Individual HPV types are categorised as low-risk and high-risk HPV types, depending on their oncogenic potential. The Lower Anogenital Squamous Terminology (LAST) guidelines were introduced in 2012 to uniformly name HPV-related lesions of the genital tract, distinguishing low-grade squamous intraepithelial lesions (LSIL) from high-grade squamous intraepithelial lesions (HSIL).[9] Vulvar lesions caused by low-risk HPV-types (formerly VIN I) are allocated to the LSIL category.[9] Lesions caused by high-risk HPV types are classified as vulvar HSIL (formerly VIN II and III). Lastly, dVIN was recognised separately in the terminology as a distinct, HPV-independent precursor lesion for VSCC. Clinical features of VSCC and its precursors are further explained upon in the forthcoming sections.

Vulvar HSIL

Vulvar HSIL is caused by high-risk HPV types, most common HPV types 16, 18 and 33.[8] The median age of onset is in a patient's 3rd and 4th decade.[10] Immune suppression and smoking are highly associated with the development and recurrence of vulvar HSIL.[11–13] The majority of affected individuals have symptoms including pruritus, dyspareunia, dysuria, pain and vulvar skin pigmentation.[13] Vulvar HSIL has a malignant potential of 3% with adequate therapy, but this may increase up to 9% when left untreated.[14,15] Only up to 1.5% of vulvar HSIL lesions are reported to regress spontaneously.[15] It is of importance to add cervical dysplasia screening during the treatment of vulvar HSIL patients, as these dysplastic conditions can coincide. Non-surgical treatment options for vulvar HSIL are immune-modulating topical therapies such as the Toll-like receptor 7 and 8 agonist imiquimod.[16,17] Reported efficacy of imiquimod are between 51—58% with 11–16% reported recurrence rates. Common side effects include erythema, irritation, ulceration and pain at application site.[18,19] Surgically, the vulvar HSIL lesions can be excised or ablated using a CO2 laser. Both procedures are characterized by painful post-operative recovery and high recurrence rates of up to 51%.[16] Studies to the efficacy of therapeutic HPV-vaccination for existing and prevention of recurrent vulvar HSIL are ongoing, with recent publications suggesting potential for patient subgroups or combinations with conventional treatment modalities.[20–22]

Future vulvar HSIL incidence will likely decline because of ongoing preventive HPV-vaccination strategies. Many nation-wide vaccination programmes have been rolled out over the past two decades to prevent cervical dysplasia caused by HPV.[23] Clear evidence for the effectiveness and justification of these programmes can now be deducted from declining incidence rates of cervical dysplasia and cancer incidence in countries that introduced vaccination in the late 2000's.[24] No significant effects have been observed for vulvar cancer or vulvar HSIL to date, which has been attributed to low disease incidence. Preventive actions notwithstanding, novel treatment options are still

urgently required to offset the numerous treatment side effects, high recurrence rates and remaining malignant potential of vulvar HSIL.

Lichen sclerosus (LS)

Patients with LS suffer from a chronic inflammatory condition that can affect non-mucosal skin with a preference for the genital area. Genital LS reportedly has a peak incidence in postmenopausal women but can also occur in men and women of all ages, including prepubertal girls. The exact aetiology of LS remains unknown.[10,25] However, an association between the incidence of autoimmune disorders and LS and a positive family history has been found, suggesting a role of immunity and genetics in the aetiologic pathway.[26–28] The classic clinical presentation of vulvar LS is a pruritic, hypopigmented 'figure of eight' area of atrophic vulvar skin, bilaterally and symmetrically reaching outward from the clitoris to encompass the labia minora, dipping inward at the perineum and reaching around the anus to form a number eight shape. The hypopigmented skin may display fissures, erosive areas, and, in advanced stages, agglutination of labia minora, concealment of the clitoris and narrowing of the vaginal introitus. VSCC can arise in affected LS tissue in small percentage patients, necessitating regular follow-up.[29,30] Pruritic symptoms can be alleviated by treatment with life-long, ultra-potent topical corticosteroids on the affected vulvar skin.[31–33] There are indications that disease progression and malignant transformation can be partially prevented by corticosteroid treatment, but prognosis is dependent on prompt and accurate diagnosis, correct treatment and patient compliance.[34] Substantial morbidity of physical, sexual and psychological nature persists despite available therapy.[35–37] This austere prospect will not change without advancement of adequate aetiologic understanding and innovative LS treatment modalities with disease-specific targets.

Differentiated VIN

HPV-independent VSCC and its precursor, dVIN, have been associated with mutations in tumour-suppressor oncogene TP53.[38,39] dVIN is frequently found with a background of LS in postmenopausal women, although dVIN lesions can also develop independently.[40] Recognition of dVIN lesions is challenging, often requiring a specialised clinician and pathologist to come to an accurate diagnosis. Patients with dVIN have a reported absolute risk of 33–86% to develop primary VSCC and 32–64% for recurrent VSCC. Cancer progression usually occurs within 2 years after dVIN diagnosis.[41,42] This rapid and high malignant potential necessitates swift treatment with radical excision and follow-up surveillance. Recurrence risks and VSCC risk are probably dependent on residual dVIN in the resection margins.[43] The first line of treatment for dVIN is surgical excision, although an onerous balance remains between complete (pre)malignancy removal and needless resection of essential vulvar tissue. The management of these patients is challenging because of the difficult lesion recognition, which demonstrates the need for advanced imaging methods to differentiate healthy from diseased vulvar tissue.

VULVAR SQUAMOUS CELL CARCINOMA (VSCC)

Vulvar cancer represents about 4% of all gynaecological cancers, occurring in 2.6 per 100.000 women per year with average 5-year overall survival rates of 71%.[44–46] The predominant histological subtype, in 85-90% of cases, is VSCC.[47,48] VSCC risk increases with age with a peak incidence at 70 years, in line with the age-bound incidence of dysplastic precursor lesions.[44] Regional metastases in the groin occur in approximately 25-30% of cases, while distant metastases are rare (5%).[49] Surgery is the cornerstone of treatment for VSCC. Surgical treatment for VSCC is associated with damage to essential structures such as the distant urethra, clitoris or anus with significant morbidity.[4,50] Despite radical surgical margins during surgery, local recurrence rates up to 40% have been reported, prompting necessity for re-excision and severely reducing survival chances.[51–54]

BENIGN HUMAN PAPILLOMA VIRUS (HPV) MEDIATED DISEASE: CONDYLOMATA ACUMINATA AND CUTANEOUS WARTS

HPV is a double-stranded virus that can cause subclinical or latent infections sequestered away from the immune system in the epithelium of cutaneous or mucosal tissue. Over 180 HPV types have been described and classified in phylogenetic groups. The Gamma, Mu and Nu phylotypes mostly cause benign cutaneous lesions, while HPV types from the Alpha genus preferably infect mucosal tissues.[55] The oncogenic potential of HPV types determines their classification as either high-risk or low-risk. Whereas the high-risk variants are the notorious drivers of cervical cancer, low-risk types can cause common skin lesions such as cutaneous warts and condylomata acuminata. HPV type 6 and 11 can cause benign anogenital warty lesions in both men and

women called condylomata acuminata, more commonly known as anogenital warts (AGW). Condylomata are highly contagious, with a reported 65% of individuals whose sexual partner also develop anogenital warts. Patients may experience psychological discomfort from the cosmetically disfiguring nature of the lesions and concerns about infecting sexual partners.[56] Cutaneous warts, also known as verrucae, are common skin lesions a reported prevalence of 3.6–22% in schoolchildren and 0.84–13% in adults.[57–61] HPV type 1, 2, 7, 27 and 57 the drive cutaneous wart formation.[62,63] Although these lesions do not have malignant potential, patients can report physical or psychological discomfort, including pain or embarrassment.[64]

Untreated or unsuccessfully treated warts, both of cutaneous and anogenital nature, pose a pool of infection on an individual as well as a community level.[65,66] Current treatment options focus on destruction of the epithelium rather than specifically targeting the HPV-infected keratinocyte.[67–70] The reported efficacy rate of treatments for AGW is 45–83% with recurrence rates of 19–77%, with side effects that may include pain, burning sensation and blistering. Clearance rates of cutaneous warts is 24–40%.[71,72] Re-emergence of the lesions is thought to be due to failure to remove the reservoir of HPV present in surrounding (anogenital) tissue.[73] The drawbacks of the current wart treatments encourage the development of novel treatment modalities with higher specificity, improved efficacy and reduced side effects. Many studies to new therapies are limited by shortfalls in objective assessments to measure effect. Accurate biomarkers to quantify disease therefore need to be embedded into these clinical trials.

Biomarkers for comprehensive disease monitoring and therapeutic target recognition

Biomarkers are quantifiable measurements of a biological process that can contribute to diagnosis, prognosis and therapy of diseases.[74] A biomarker can reflect the normal or pathological process or the pharmaceutical response to a therapeutic intervention.[75] A classic example of a biomarker in gynaecologic oncology is the immunohistochemical P16 staining as a surrogate marker for high-risk HPV infection in biopsy tissue of cervical lesions.[76] In drug development, biomarkers are increasingly being applied to predict clinical benefit or efficacy of a new compound and to substitute for a clinical endpoint in case of chronic and long-term diseases. Objective and sensitive clinical endpoints need to be recognised and tested for validity prior to application in clinical trials. Validation of biomarkers in a structured manner is based on five pillars: repeatability, tolerability, discriminatory capacity between diseased and healthy characteristics, treatment effect and correlations to traditional endpoints.[77] By incorporating precise and accurate biomarkers in early-phase clinical studies, compounds with limited efficacy can be abandoned in favour of more promising ones, saving precious economical resources and reducing the amount of patients exposed to ineffective treatments.[78,79] Additionally, with the growing understanding of the complexity of diseases, biomarkers will play an increasing role in personalised therapy choices. Especially in vulvar diseases such as VSCC, vulvar HSIL, dVIN and LS, there is a lack of accurate, precise and non-invasive biomarkers to guide diagnostics, clinical follow-up and treatment effects.

Recognition of novel biomarkers can be approached in a structured manner encompassing multiple domains (*Figure 3*).[80] A multitude of domains ranging from histological findings to patient reported outcomes may generate potential candidates to be used as biomarkers in future applications. Incorporating new, tailored biomarkers in clinical trials in turn depends on the pharmacokinetic and pharmacodynamic considerations of potential drug candidates. Clinician-based scoring of vulvar disease can be complemented by symptom reporting by patients and e.g. by wearables measuring scratching as a surrogate biomarker for pruritus.[81] In addition, non-invasive techniques such as biophysical assessments (e.g. pH, trans-epidermal water loss) can be applied to acquire an insight on the biological process at the surface of the vulvar skin. Imaging techniques have advanced significantly over the past decades with increasing resolution and quantifiable measurements in different skin layers. Histology and immunohistochemistry can provide insight in mechanistic processes on a cellular level, which can be complemented by molecular sequencing techniques to uncover genomic properties and gene expression profiles. When considering application of topical medication, pharmacokinetic properties of the compound through the skin to the intended target is of utmost importance, as the skin's primary function is to provide a barrier to external factors. Finally, there is increasing evidence that the microbiome composition plays an essential role in health and disease, and its interaction should be further understood. All these domains together can provide a holistic, phenotypic reflection of a disease entity that can be applied to improve disease recognition and

understanding and to guide therapeutic target development. This thesis will extensively focus on two domains from this comprehensive approach:

1 non-invasive **imaging** for disease recognition and follow-up
2 the vulvar **microbiome** composition.

These primary categories of interest are accompanied by components from the other areas highlighted in the flower-shaped model.

ADVANCED IMAGING TECHNIQUES FOR DISEASE RECOGNITION AND FOLLOW-UP

The dermatology practice routinely applies imaging in for improved visualisation of cutaneous lesions and as a follow-up reference.[82] Imaging techniques have advanced significantly over the past decades, with increased resolution, improved follow-up possibilities and expanded quantifiable measurements in various skin layers.[83] These developments reduce the need for relatively subjective clinical scoring systems. Consecutive, non-invasive measurements can be obtained whilst providing objective data over time, as opposed to limitations posed for invasive methods such as biopsies. Clinical imaging of vulvar diseases in the daily gynaecological practice is currently performed as photo-documentation for follow-up, generally using a conventional photo camera system, despite the challenges in the recognition of vulvar disease such as vulvar HSIL and dVIN. In this thesis, several potential imaging systems are applied in a clinical trial setting to assess their potential application for the vulvar clinic. These include dermatoscopy, optical coherence tomography (OCT) and reflectance confocal microscopy (RCM) and stereophotogrammetric three-dimensional (3D) photography (*Figure 4*).

Dermatoscopy has been integrative part of the dermatology practice for many years. The diagnostic accuracy of the clinical evaluation potentially malignant lesions transformation has greatly improved since the widespread utilization of dermatoscopy systems.[84–86] These dermatoscopy systems can range from simple, hand-held magnification glasses to vast, high-resolution cameras linked to analysis programmes including lesion location options and follow-up functionalities. Dynamic OCT (D-OCT) is most renowned for being the clinical standard in the ophthalmology clinic for the diagnostics of retinal diseases through visualization and quantification of the retinal microcirculation.[87,88] D-OCT uses light reflectance to generate a real-time black and white image perpendicular showing the imaged skin and its components up to a depth up to 2.0 mm. The analysis of these visualised skin layers can be used to determine epidermal thickness, cutaneous blood flow and skin roughness.

This technique has been applied in dermatology research for the assessment of skin thickness and cutaneous microcirculation, but more sparingly on the vulvar area.[89–94] RCM applies a low powered laser (830 nm) to generate a non-invasive and real-time visualization of the skin (up to a depth of 150 µm) with cellular resolution.[95] The technique has proven useful as adjunct tool for the recognition and diagnosis of malignancies of the skin and may reduce the need for biopsies.[96,97] Finally, stereophotogrammetric 3D photography is a hand-held camera system that is already utilized in the field of plastic surgery in order to add objective measuring techniques to the clinical practice.[98,99] Stereophotogrammetry obtains two or more images from different angles which can subsequently be reconstructed into a 3D image. The technique has previously been used in the assessment of scars, but not for the study of vulvar diseases.[100,101]

THE MICROBIOME AND ITS ROLE IN GYNAECOLOGICAL HEALTH AND DISEASE

The microbiome, the aggregate of bacteria, viruses and fungi in a niche, plays a key role in human health.[102] Alterations in the microbiome composition have been associated with several conditions, including inflammatory disease, skin conditions and cancer.[103,104] Dysbiosis of the whole microbiome composition in an anatomical niche is believed to drive DNA damage, inflammatory responses and aberrant signalling pathways summating to tumorigenesis and cancer progression.[105] Therefore, the maintenance of an optimal microbiota composition could prove crucial in cancer prevention.

Large-scale studies to the cervical and vaginal bacteriome composition have described relationships between microbiome changes and pre-term birth, bacterial vaginosis and cervical dysplasia. Of these, the vaginal microbiome has most extensively been studied (*Figure 5*). The vaginal bacteriome of healthy women is characterized by inter-individual heterogenicity and temporal variability, which can partially attributed to demographic characteristics and lifestyle.[106,107] *Lactobacillus* is a genus of Gram-positive anaerobic bacteria that are often identified in the female genital tract, which preserve an acidic vaginal milieu. Patterns of the vaginal microbiome composition have been clustered into numbered community state types (CST).[108,109] The vaginal CST I is dominated by *L. crispatus*, CST II by *L. gasseri,* CST III by *L. iners* and CST V by *L. jensenii*. The non-*Lactobacillus*-dominated CST IV constitutes a diverse pattern with strictly anaerobic bacteria, such as *Atopobium vaginae*, *Gardnerella vaginalis* and *Megaspheara elsdenii.*[109] These bacteria have

also been associated with bacterial vaginosis.[110,111] The complete microbiome encompasses more than just the bacteriome, although most of the research has focused on this dominant component. Current knowledge on the mycobiome, the fungal fraction, is primarily derived from culture-based studies. In gynaecology, candidiasis is a well-known opportunistic fungal infection of the vaginal tract that can cause pruritic symptoms and purulent discharge. The causative fungus, *C. albicans*, colonises up to 20% of women asymptomatically, rendering it a commensal with pathogenic properties.[112] The viral fraction of the vaginal microbiome composition is even less extensively studied, except for the role of HPV on dysplasia and cancer.

Gynaecological oncology covers malignancies of all female reproductive organs, including the cervix, vagina, uterus, ovaries, fallopian tubes and vulva. The cervicovaginal microbiome in relation to cancer has been studied extensively.[113] A high-diversity cervicovaginal microbiome composition and disappearance of *Lactobacillus* dominance associates with advancing stages of HPV-driven cervical dysplasia.[114–116] In addition, increased prevalence of vaginal *Snaethia* spp. has been described in patients with hrHPV infections, cervical intraepithelial neoplasia and invasive cervical carcinoma.[117–120] These findings endorse a potential causal link between vaginal dysbiosis and cervical cancer, influencing cancer development in all phases from acquisition and persistence of HPV to formation and progression of cervical dysplasia.[116] Vaginal dysbiosis may also drive ovarian or endometrial cancer through immunogenic disruption.[121] In endometrial cancer, presence of *Atopobium vaginae* and *Porphyromonas* species and significantly elevated levels of *Proteobacteria* and *Firmicutes* phylum bacteria have been reported. *Chlamydia trachomatis*, *Lactobacillus* and *Mycobacterium* have been suggested to potentially influence ovarian cancer development.[122]

Although patterns of microbiome disruptions have been studied for other gynaecological cancers, no studies have been conducted that investigate the vulvar microbiome in relation to vulvar cancer or its precursors. Apart from a handful of culture-based studies conducted in the previous century, there seems little interest into understanding the healthy vulvar microbiome composition.[123–125] Insights in the healthy microbiome composition is the basis to correlate alterations to disease states. Our knowledge of the intestinal and vaginal microbiome indicates a pivotal role of the microbiome in the pathway to malignancy. The lack of interest in translation of this expertise from other anatomical locations to the vulvar research field is remarkable, especially considering the prominent role of a microorganism, HPV, in the development of vulvar HSIL and VSCC. Additionally, we have no clear understanding of LS aetiology, nor conclusive evidence explaining disease progression to dysplasia and VSCC. In conclusion, the vulvar microbiome composition is an untapped field of research with myriad possibilities to expand vulvar disease understanding. Awareness of the role of the microbiome of the vulva may unlock opportunities to improve disease recognition and development of therapeutic options.

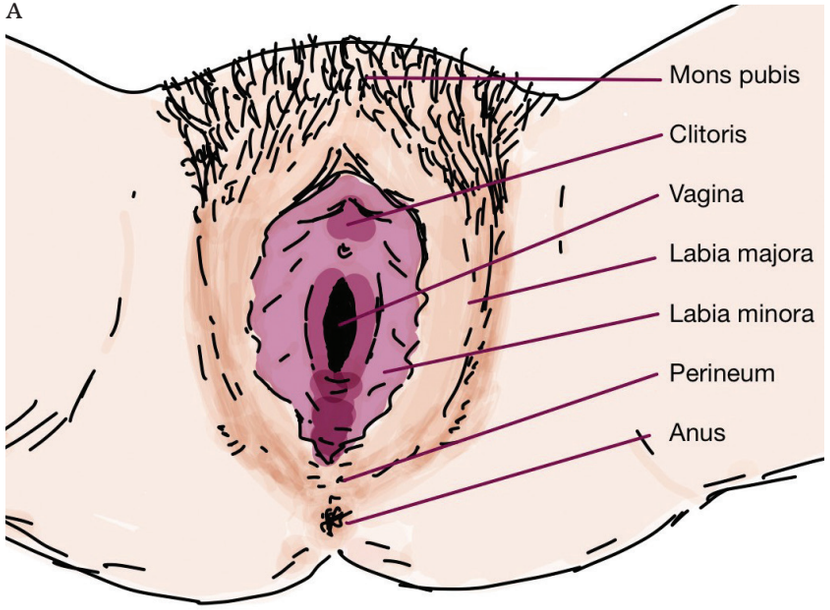
Aims and outline of this thesis

The aim of this thesis was to study novel tools and biomarkers for improved detection of vulvar premalignant disease and aid the investigation of potential new drug targets for the indication of vulvar and HPV-driven diseases.

Section I of this thesis focuses on the recognition and validation of imaging-based biomarkers for HPV-driven diseases of the skin and external genitalia. In *chapter 2*, stereophotogrammetric photography was studied for the use in cutaneous warts, vulvar HSIL and AGW as reliable photo-documentation and lesion size calculation. *Chapter 3* presents a pilot study for the application of dermatoscopy and optical coherence tomography (OCT) for the visualisation and characterisation of vulvar HSIL and LS. In *chapter 4*, reflectance confocal microscopy (RCM), which allows for detailed dermal imaging at cellular resolution, was applied in the same patient population. In *chapter 5*, a first-in-its-class small molecule was tested in a Phase I trial for safety and exploratory efficacy for the treatment of cutaneous warts, using stereophotogrammetric 3D photography and OCT as exploratory biomarkers.

Section II investigates sequencing-based biomarkers for vulvar disease, facilitating insight into the aetiology of vulvar diseases and identifying potential new therapeutic targets. The currently available literature on the vulvar microbiome composition was investigated in *chapter 6*. The findings from this review of literature are further expanded in an observational study characterising the vulvar microbiome composition of healthy volunteers, vulvar HSIL patients and LS patients, which is described in *chapter 7*.
Lastly, *chapter 8* summarises the findings of this this thesis with a call to the research field to improve the biomarkers for vulvar HSIL and LS. In addition, a perspective on recent developments in the field of premalignant vulvar disease is provided, with recommendations for future applications of the biomarkers studied in this thesis.

Figure 1 Schematic overview of vulvar anatomy. A) overall anatomy and B) histological features, signifying the transitional skin types from the inguinal area, the labia majora, labia minora to the vaginal mucosa. Figures made using Apple GoodNotes 5 and Biorender.



(continuation Figure 1)

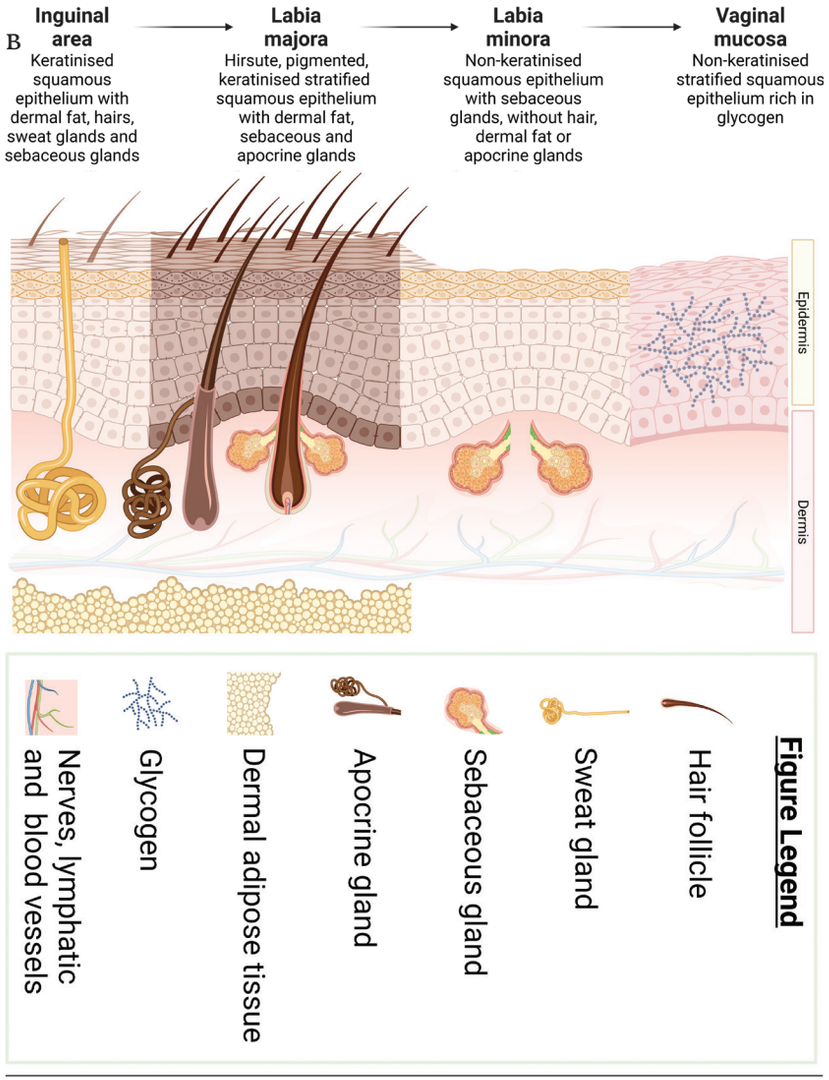
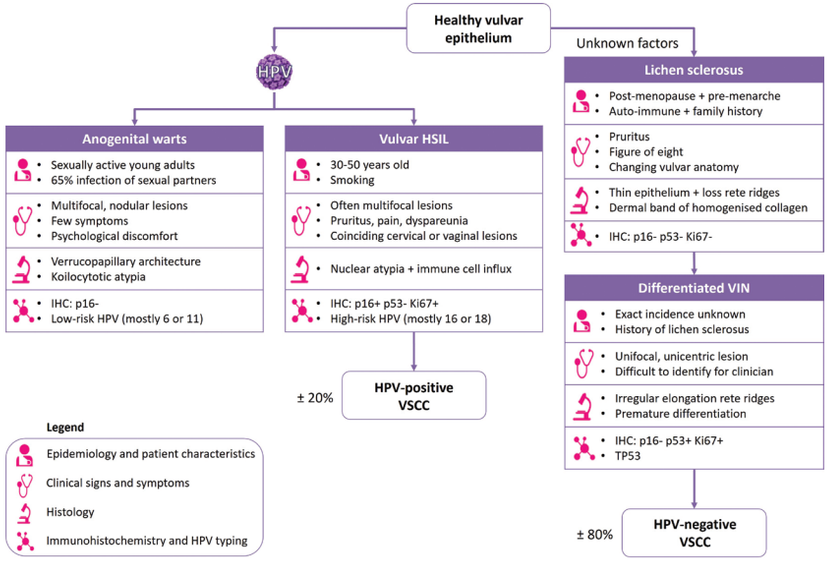
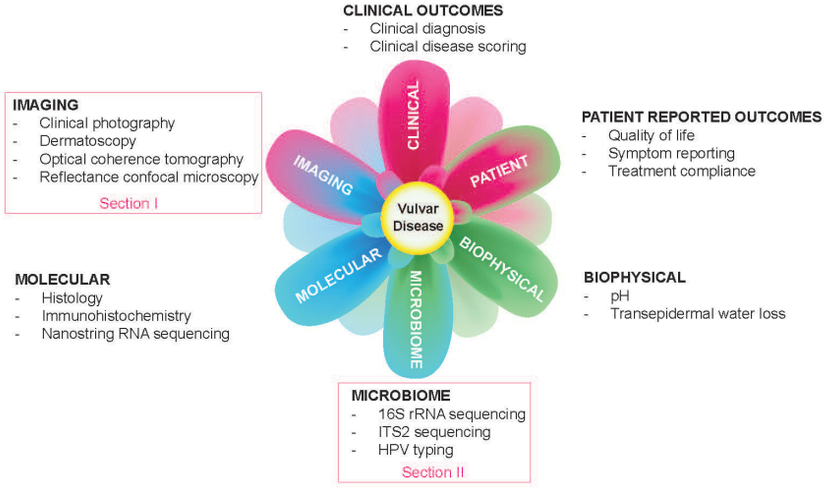


Figure 2 Human papillomavirus (HPV)-independent and HPV-dependent pathways leading to distinct subtypes of vulvar lesions and vulvar squamous cell carcinoma (VSCC).



HSIL = high-grade squamous intraepithelial lesions, VIN = vulvar intraepithelial neoplasia, IHC = immunohistochemistry, HPV= Human-papillomavirus, TP53 = Tumour protein P53.

Figure 3 Multi-modal approach to characterising (vulvar) diseases.



RNA = Ribonucleic acid, ITS2 = Internal transcribed spacer region, 2 HPV = Human-papillomavirus

Figure 4 Imaging techniques. Examples of novel imaging techniques applied in this thesis, showing in clockwise fashion in ascending order of magnification and resolution stereophotogrammetric three-dimensional (3D) photography, dermatoscopy, optical coherence tomography (OCT) and reflectance confocal microscopy (RCM). Figure made using BioRender.

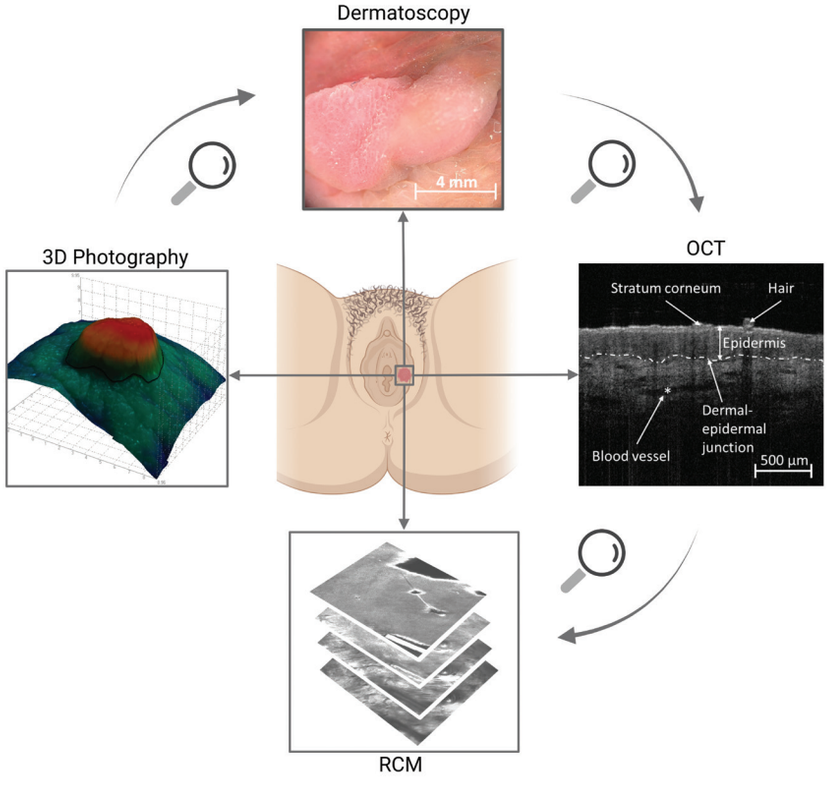
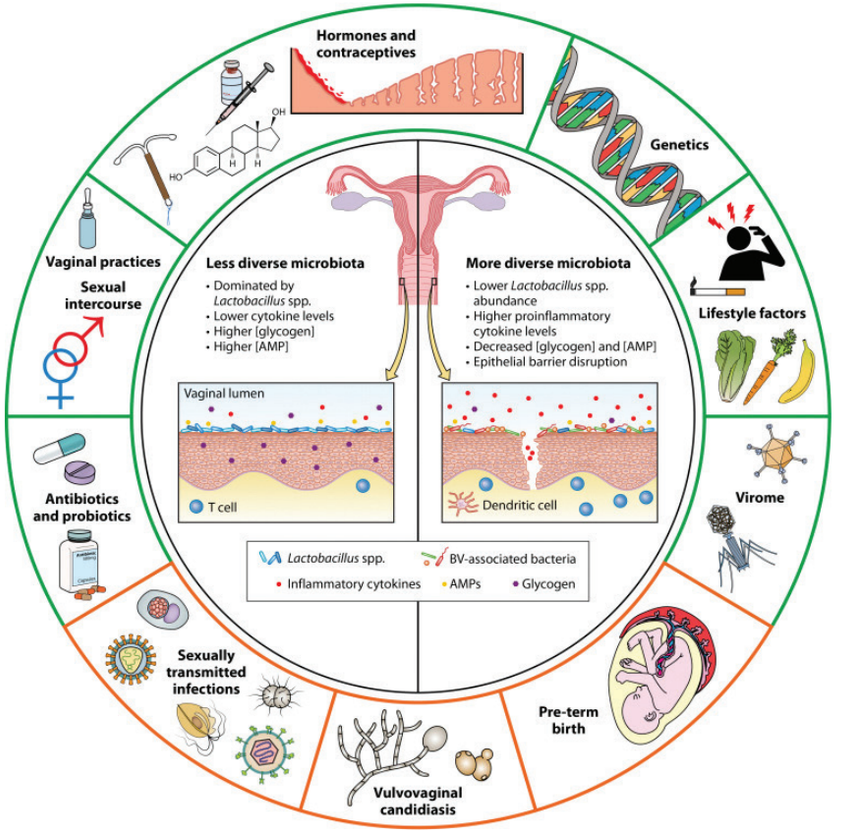


Figure 5 The vaginal microbiome composition and correlations to disease. External and internal factors that have been shown to influence the vaginal microbiome composition and its consequences on a functional and clinical level. Reproduced with permission from Dabee *et al*, 2021.[113]



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