Characterization of vulvar diseases: novel imaging tools, models and molecular targets

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CHAPTER 7

General discussion

As discussed in the introduction of this thesis, patient burden from vulvar (pre)cancers remains high despite evolving care.[1] This burden arises partly due to: I) problems in the diagnostics process, and II) as a result of the treatment regime. The diagnostic process is impeded by incorrect or delayed diagnosis. This is in part a result of the challenges accompanying a physician's visual and tactile assessment of vulvar diseases.[2] Additionally, invasively obtained biopsy material for pathologic review is limited and sometimes inconclusive. Delays may also occur when patients misconstrue symptoms due to lack of awareness or shame.[3,4] Patient burden may also arise as a result of the treatment regime, side effects or insufficient impact of treatments.[5–7] Luckily, the number of novel instruments has expanded, providing many new possibilities for value-based objective endpoints and biomarkers.[8]

In the present thesis, we have attempted to test several promising innovative instruments in a search for disease-specific biomarkers for vulvar (pre)cancers. A multimodal profiling approach (Figure 2, **Chapter 1**) guided this research, based on systems profiling of disease and drug effects in dermatology.[9] The different investigated aspects include imaging, cellular and molecular characteristics and was complemented by patient reported outcomes and physician-based input. The focus of this thesis has specifically been on the molecular and imaging domains. It was assessed if the techniques included in these domains show the potential to improve the diagnostic process and/or the evaluation of therapeutic options for vulvar (pre) cancers. Over the longer term, the goal is to improve the burden for patients with vulvar (pre)cancers. This chapter summarizes and discusses the results and highlights perspectives for future research.

As previously discussed, FIGO staging and diagnosis of vulvar (pre)cancers still depends on clinical examination and histopathologic review of invasive biopsy material.[10] In comparison to other more common cancer types, the literature concerning non-invasive imaging for identification and diagnosis of vulvar (pre)cancers is sparse and based mainly on case reports.[11–15] Vulvar lesions are easily accessible as they originates from the skin. Therefore, various non-invasive imaging tools were examined in patients with vulvar high grade squamous intraepithelial lesions (vHSIL) and lichen sclerosus, seeking for sensitive disease-specific biomarkers (**Chapter 2 and 3**). Using three different innovative non-invasive techniques, the vulvar skin was assessed at different skin levels, from superficial up to 2mm in depth. Respectively dermatoscopy[16], optical coherence tomography (OCT)[17] and reflectance confocal microscopy (RCM)[18] were examined. Dermatoscopy uses a lens with a build-in light source to magnify the skin surface in the transversal plane. OCT is comparable to ultrasound but uses laser light instead of sound waves for image acquisition. OCT creates images in the frontal plane, up to a depth of 1mm. RCM uses a diode laser as light source that illuminates the skin and gets reflected differently by various cell types. Reflection of cells is based on their refractive indices; this generates black-and-white images with a resolution almost comparable to conventional histology. RCM enables real time *in vivo* visualization of the epidermis down to the papillary dermis (2mm in depth) in the transversal plane.

It was demonstrated that the application of dermatoscopy, OCT and RCM was feasible and tolerable in vulvar high grade squamous intraepithelial lesion (vHSIL) and lichen sclerosus (LS) patients as well as in healthy controls. Of the three non-invasive innovative imaging tools dermatoscopy was considered the easiest to implement in clinical practice (**Chapter 2**). The execution is straightforward, the assessment time is short and interpretation comes with frequent use. Analysis of a set of dermatoscopic characteristics revealed that warty structures were only observed in vHSIL. In LS, sclerotic areas and arborizing vessels appeared as potential discriminative features. The dermatoscope is also lowest priced compared to the OCT and RCM. The prices of this instrument can be as low as 1.000 United States Dollars (USD), while the newest integrated systems including data capacity, may cost 10.000 USD. Dermatoscopy could ease diagnostics and follow-up and help as a guidance tool for biopsy sampling of suspected vulvar lesions. This technique could for example be of great advantage for imiquimod-treated HSIL patients, as their follow-up requires long-term surveillance of the entire genital tract because of the high probability of late recurrences.[19] This is particularly important to diagnose malignant progression, which is currently a challenge for clinicians. Consequently, patients with other (pre) malignant vulvar diseases as dVIN and VSCC should be included in larger follow-up clinical trials. Several studies have confirmed that the diagnostic accuracy of pigmented lesions and the diagnostics of melanomas, is significantly better with dermatoscopy compared to conventional diagnostics if performed by an experienced user.[16]

We showed alignment is possible between some morphological disease features assessed and histopathology, although time-consuming. Primary focus was on the incorporated (automatic) OCT algorithms, measuring epidermal thickness and blood flow. It was concluded that these algorithms should first be adapted for the vulvar skin prior to potential implementation (**Chapter 2**). Although an OCT blood flow measurement on itself is currently unable to discriminate different vulvar diseases, it might be used as additional biomarker in a multimodal approach. It could hereby aid in the differentiation of healthy and diseased vulvar skin with comprised blood vessels like in inflamed tissue.

RCM images showed that healthy skin was characterized by a homogenous, normal honeycomb patterned epidermis and a clear epidermal-dermal junction. Vulvar HSIL and LS lesions often displayed an atypical honeycomb pattern of the epidermis, combined with lymphocytic influx and the presence of melanophages. Distinct features specifically observed in LS included the presence of hyalinized vessels and sclerotic areas in the dermis (**Chapter 3**). Due to the explorative nature of this study and small sample size, no statistics were performed on these characteristics. RCM appears to have potential as optical biopsy tool but requires clinical validation in larger patient groups with long-term follow-up before implementation. Future studies should keep evaluating existing and emerging non-invasive imaging techniques developed for the discrimination of malignant progression of vulvar lesions. Preferably imaging tools are integrated in one device to acquire images at different skin depths.[20]

Besides the advantage of being easily accessible from the outside, this vulvar cancer type originates from squamous cells, just like cutaneous squamous cell carcinoma (CSCC) does. This allows for knowledge transition between the dermatologic and gynecologic fields. For instance, in CSCC, epidermal homeostasis gets disrupted by interactions between epidermal tumor cells and the underlying stroma, allowing epidermal cells to invade into the stroma [21]. This process for CSCC is being studied in human-skin-equivalents (HSE's). HSE's are *in vitro* cultured skin models designed to mimic the characteristics of human skin as closely as possible. These models are used as tool to study biological processes in healthy and diseased skin.[22,23] Moreover, HSE's can serve as a prediction model to determine the penetration profile of drugs across the skin.[24] As one of the aims of this thesis has been to improve new therapeutic options for vulvar (pre)cancers, HSE models have been developed that mimic healthy and malignant vulvar skin (**Chapter 4**). Remnant skin specimen obtained at labial corrections were used to set up HSE models of the vulva. Vulvar keratinocytes were isolated, pre-treated and 'seeded' on a base of fibroblast cells in a petri dish. Comparison of the epidermal morphogenesis of normal vulvar tissue in these models showed great similarities with native healthy vulvar tissue. Immunohistochemical analysis using keratin-10 expression showed a physiological early cell differentiation program in the healthy models. The expression of keratin-17 is known to be absent in healthy epidermis and likewise not detectable in the models, stipulating the inactivated state of the keratinocytes. In conclusion, immunohistochemical results showed similar expression in the healthy HSE models compared to native vulvar tissue.

To allow pathophysiological characteristics to be easier transposed to clinical observations, tumorous HSE models were developed and compared to the healthy HSE models. For the set-up of VSCC models immortalized VSCC cell lines HTB117 and A431 were grown on a dermal scaffold of different fibroblasts. Both papillary- and reticular fibroblasts (RF) were used for dermal scaffolds, as these fibroblast types are generally present in healthy stroma. Also, dermal scaffolds with cancer-associated fibroblasts (CAF's) were generated, as this heterogenous cell is known to be part of the tumor microenvironment (TME) of different SCC's.[21,25–27] All different tumorous HSE models showed typical SCC progression and revealed the interplay between VSCC cells and tumor stroma within VSCC development. CAF's in the CAF-based models expressed a consistent high level of α-SMA and COL11A1 after interaction with VSCC cells. This indicated that the phenotype of CAF is maintained, which might partially explain the profound invasive behavior observed in these models. A loss of RF markers and a gain of CAF marker expression was observed in the RF based models, indicating the apparent transition of a RF- into CAF phenotype. This shows that communication between VSCC cells and stromal fibroblasts could change a fibroblast's phenotype.[27] Recent studies already highlighted the importance of the immune TME involvement for VSCC's.[28,29]

These models thus aid in gaining pathophysiological knowledge and can also be used for non-clinical drug evaluation. Non-clinical drug testing serves as a basis for the evaluation of potential risks and effectiveness of investigational drugs in humans.[30] If the novel vulvar tumor models are qualified and validated, they could offer great potential for research on drug action. [30–33] The information obtained can be used to design trials that are

well-informed, addresses the pertinent questions and will most likely require smaller populations. This could alleviate the difficulties recruiting patients for clinical trials due to the low disease-incidence rates. The models could help improve the treatment of vulvar (pre)malignancies as hardly any significant new topical agent after imiquimod has been developed in the last decades for these diseases. Additionally, with the low number of leads on chemotherapeutic and targeted antibody treatments in the vulvar cancer niche, availability of these models could be an enormous step forward in early clinical drug development for VSCC.[34] A first step in testing drug response as part of this paradigm was done by adding chemotherapeutic agents carboplatin and paclitaxel to the medium of the VSCC models. A dose-dependent reduction of the relative tumor thickness was observed in the models (**Chapter 4**). Reduction of the epithelial cancer cells was most pronounced in the paclitaxel group. Remarkably, chemotherapeutic treatment did not seem to affect the expression intensity of A-SMA (CAF marker) in the VSCC models. This may corroborate the suggestion that CAF's can REPROgram tumor cell metabolism to maintain tumor cell survival and protect them from apoptosis induced by treatment. It further confirms the hypothesis of others reporting on 3D VSCC models created with CAF's and a self-established cell line of vulva cancer associated with lichen sclerosus.[35] The fact that CAF's remained *in situ* after chemotherapeutic treatment with carboplatin or paclitaxel is a major learning point, and emphasizes the need for drugs targeting stromal fibroblasts within the TME.

For an improved representation of the diversity of vulvar tumors and their TME, the organotypic 3D models could be personalized with isolated tumor cells of a patient. Research using patient-derived tumor organoid (PDTO) models has shown to recapitulate the molecular and phenotypic heterogeneity of the patient tissue they were derived from.[36] Recent studies have shown the ability of these PDTO'S to predict patient drug response. For example, PDO models were used to predict responses for a chemotherapeutic in more than 80% metastatic colorectal cancer patients.[37] We suggest that personalized 3D vulvar models prove an attractive and scalable tool for drug screening, as they allow for expansion and biobanking. Having personalized targeted treatment options might greatly improve the flexibility and personalization of vulvar cancer care in clinical practice, as shown for e.g. psoriasis.[9] Ideally, these personalized vulvar tumor models are used in a multimodal matter. Besides the use for drug evaluation of existing treatments, the vulvar 3D models might be used for pathway- and drug analyses on somatic mutations for novel specific therapeutic strategies.[38,39] Also, healthy and tumorous vulvar models can be used for molecular profiling for tumor-specific imaging. The latter includes all techniques used for real-time highlighting of tumors. One type of tumor-specific imaging is applied in the emerging field of fluorescent-guided surgery (FGS). FGS makes use of a contrast agent consisting of an antibody, small synthetic molecule, aptamer or a nanoparticle bound to a fluorescent label able to highlight the tumor. FGS real-time assists the surgeon with the identification of the tumor and the usage of near-infrared light allows for deeper tissue penetration. Ultimately, improved tumor margin detection using FGS could enhance precise surgery and limit morbidity. As this technique could potentially contribute to both improved diagnostics and treatment of vulvar (pre)cancers, it was further investigated in **Chapter 5 and 6** of this thesis.

A first step for implementation of targeted FGS, is the identification of markers that bind specific to vulvar carcinoma cells. A literature review was performed in **Chapter 5**, to identify potential tumor markers that could be used for FGS of differentiated vulvar intraepithelial neoplasia (dVIN), vHSIL and VSCC. A total of 627 articles were found, of which 222 met all eligibility criteria. Twelve vulvar carcinoma-specific tumor targets were identified and from these, 7 were considered most promising: EGFR, CD44V6, GLUT1, MRP1, MUC1, CXCR-4 and VEGF-A. However, most of these papers did not describe expression of these cell membrane located proteins in healthy vulvar tissue. Whilst this aspect is of key importance for the tumor-to-background ratio required to identify the aberrant tissue with FGS. Therefore, the expression of these targets should be tested in both healthy and (pre) malignant vulvar tissues. To investigate if the markers mentioned in literature are indeed candidates for FGS, the presence of these proteins has been examined by immunohistochemistry in healthy and diseased vulvar tissue samples (**Chapter 6**). Besides these literature-based markers, potential targets for FGS in other types of cancer were also included in this analysis. This resulted in the expression analysis of the integrin $\alpha v\beta 6$ ($\alpha v\beta 6$), CAIX, CD44V6, EGFR, EPCAM, FRα, MRP1, MUC1 and UPAR. The immunohistochemical presence of each marker was quantified using digital image analysis. Results showed that $\alpha v\beta 6$ was significantly overexpressed in malignant cells compared to healthy vulvar cells. Tumor to background ratios for

αvβ6 in premalignancies dVIN and HSIL were lower compared with VSCC, but still above a set threshold. Higher αvβ6 expression was found in HSIL adjacent to HPV-dependent VSCC's compared with isolated HSIL, encouraging more effective removal of adjacent HSIL during VSCC surgery. The potential of αvβ6 was also confirmed in a within-patient analyses of each individual tissue slide of a VSCC patient, including both healthy and malignant tissue. CAIX, EPCAM, MRP1 and FRα showed no or low expression in vulvar malignant tissues and were therefore excluded from further evaluation. CD44V6 and EGFR showed an overall high expression in all tissues, including normal squamous epithelium. MUC1 and UPAR were excluded from further evaluation due to their heterogenous or patchy expression pattern in vulvar malignant tissue.

Further validation of αvβ6 as a potential target for FGS of vulvar (pre) cancers requires marker expression testing in vulvar animal- or 3D organotypic models. The vulvar models described in **Chapter 4** might be used to test promising targets. To do so, a contrast agent targeted against a promising target could be added to the medium of both healthy and tumorous *in vitro* vulvar models, whereafter expression rates are examined and compared. In this way, intravenous administration of a contrast agents is mimicked *in vitro*. Besides this imitation of intravenous administration, other application routes of contrast agents or drugs can be assessed. The effect of a contrast agent could be tested if incorporated into a cream or spray and applied to the surface of the vulvar skin of the air-liquid based 3D models. Another example: 5-ALA could topically be applied on the surface of the vulvar tumor models as non-targeted photodynamic therapy. This way, the models can help test for resistance to 5-ALA, a problem currently experienced in treatment of vulvar intraepithelial lesions with this drug.[40] Also targeted imaging can be combined with treatment. This requires conjugation of a tracer to a label compatible for treatment and imaging purposes. For example, with an αvβ6 specific knotting peptide conjugated to e.g. FDA approved indocyanine green.[41,42] This contrast agent might be used for both imaging of the targeted lesion and as treatment; by killing of tumor cells trough the production of singlet oxygen species and photothermal heat upon near infrared irraditation.[43,44] Besides for fluorescent imaging, targeted imaging agents can also be generated with e.g. nuclear- or activatable labels. This enables deployment of valuable targets for many state-of-the-art real time imaging techniques. Illustrated in the brain tumor imaging niche, proving great progress in dual- or triple-modal imaging.[45] This multimodal approach makes use of e.g. FGS, MRI, CT, and/or photoacoustic imaging. A cocktail of labels coupled to a tracer targeting the same tumor target is used for this approach. The bundled merits of these techniques, like high spatiotemporal resolution, might overcome the challenges faced during single-modal imaging. Superiority of a multimodal approach in the gynecologic niche is illustrated by non-targeted dual-imaging of sentinel lymph nodes in patients with VSCC. A radioactive and fluorescent tracer (ICG-99mTc-nanocolloid) is used for improved visualization of affected lymph nodes.[46]

In the clinic, molecular VSCC targets could also be used for a pre-treatment PET signal check to predict responses in patients. A clinical study using PD-L1-targeted 89Zr-atezolizumab imaging confirmed that clinical responses in patients with different types of cancer showed increased correlation with pretreatment PET signal compared to IHC or RNA-sequencing based predictive biomarkers.[47] In addition, the search for potential VSCC targets could be extrapolated to the TME or angiovascular environment of the tumor. Imaging of the TME using nuclear medicine-based targeted molecular imaging allows for sensitive visualization of dynamic changes in the TME.[48] A nice bridge to the apparently treatment-resistant CAF's observed in the TME of our vulvar tumors in the 3D models (**Chapter 4**). Personalized tumor targeted applications using molecular biomarkers therefore show great potential. Tumor targeting could help dynamic and quantitative visualization, but also play a role in patient stratification and treatment monitoring for targeted therapy.

Overall conclusions

In this thesis, multimodal research into vulvar (pre)cancers was described by focusing on three important cornerstones:

1. Application of novel non-invasive imaging instruments on healthy and diseases vulvar skin to improve diagnostics;
2. Establishment of *in vitro* healthy and VSCC 3D models aimed for future anticancer drug evaluation studies;
3. Investigation of promising targets for tumor-specific molecular real time imaging of vulvar (pre)cancers, to assist complete surgical excision.

First steps are taken into the clinical translation of these novel techniques for the management of patients with vulvar (pre)cancers. Future assessments on accuracy, sensitivity and specificity of these techniques should be performed. For the vulvar HSE models, endpoints should be evaluated for their predictive value on *in vivo* effects and translatability to humans.

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